

The background of the entire page is a light blue gradient. It features a network of thin, light blue lines connecting various-sized, semi-transparent blue spheres. The spheres have a glossy, 3D effect with highlights and shadows. The lines and spheres are scattered across the page, creating a molecular or network-like structure.

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Cover all bases

RNA-Seq V2 Library Prep Kit with UDIs

User Guide

Catalog Numbers:

171-174, 176 (CORALL RNA-Seq V2 Library Prep Kit with UDI 12 nt Set A1, A2, A3, A4, or A1-A4)

175 (CORALL RNA-Seq V2 Library Prep Kit with UDI 12 nt Set B1)

177-180, 182 (CORALL mRNA-Seq V2 Library Prep Kits with UDI 12 nt Set A1, A2, A3, A4, or A1-A4)

181 (CORALL mRNA-Seq V2 Library Prep Kits with UDI 12 nt Set B1)

183-184 (RiboCop (HMR V2) and CORALL Total RNA-Seq V2 Library Prep Kit with UDI 12 nt Set A1 or B1)

185-186 (RiboCop (HMR+Globin) and CORALL Total RNA-Seq V2 Library Prep Kit with UDI 12 nt Set A1 or B1)

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For any publication using this product, please refer to it as Lexogen's CORALL™ RNA-Seq V2 Library Prep Kit with UDIs.

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1. Overview

Lexogen's CORALL RNA-Seq V2 Kits 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. Lexogen's 12 nt Unique Dual Indices (UDIs) are designed to provide superior error correction, accuracy, and highest quality sequencing data. The CORALL Total RNA-Seq V2 and mRNA-Seq V2 Library Prep Kits with UDIs include the Lexogen UDI 12 nt Unique Dual Index Sets A1, A2, A3, and / or A4, or B1.

For the CORALL RNA-Seq, input RNA can be either depleted of ribosomal RNA (rRNA), poly(A)-enriched, or total RNA without prior depletion or enrichment. We recommend using Lexogen's RiboCop rRNA Depletion Kits (Cat. No. 125 - 127, 144, 145, 190), bundle options are available with RiboCop HMR V2 (Cat. No. 183 - 184) and RiboCop HMR+Globin (Cat. No. 185 - 186). For mRNA sequencing, we recommend the CORALL mRNA-Seq V2 Kits (Cat. No. 177 - 182) which include Lexogen's 12 nt UDIs and the Poly(A) RNA Selection Kit upstream of the CORALL library preparation protocol. Information regarding input RNA requirements can be found in Appendix A, p.27.

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. This distance is modified using either one of the two reverse transcription buffers optimized for shorter or longer insert sizes. 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's 12 nt UDIs 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 E, p.36). All purification steps are based on magnetic beads, rendering the protocol highly suitable for automation.

CORALL RNA-Seq V2 libraries are compatible with single-read and paired-end sequencing on Illumina instruments. Read 1 contains the UMI and represents the transcript sequence. Hence the UMI information is directly accessible in 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 F, p.38.

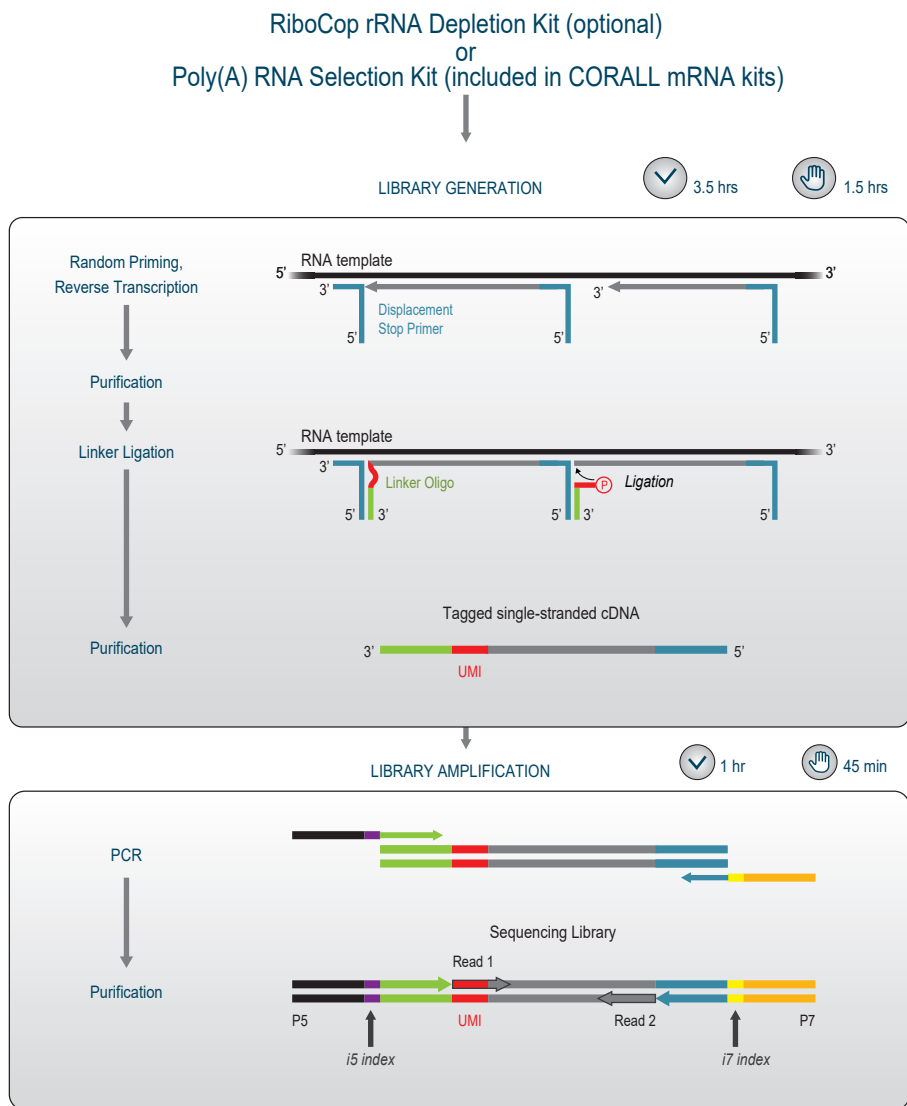
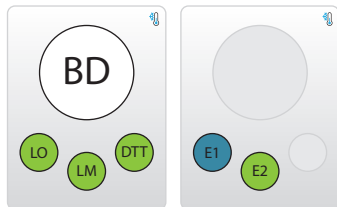


Figure 1. Schematic overview of the CORALL workflow. Bundles for CORALL RNA-Seq V2 including RiboCop rRNA Depletion Kits or Poly(A) RNA Selection Kits are available for convenience.

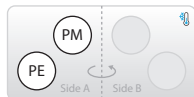
2. Kit Components and Storage Conditions

A)

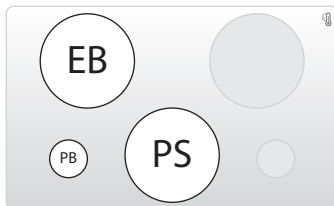
CORALL Library Generation Module, 24 reactions (-20 °C)



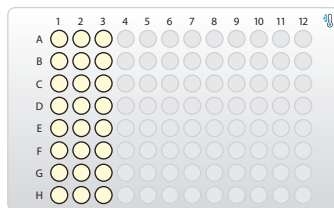
Library Amplification Module, 24 reactions (-20 °C)



Purification Module 022.24 (+4 °C)

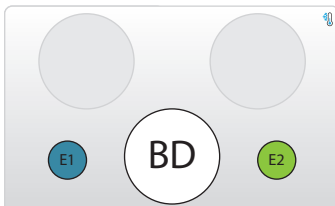


Lexogen UDI 12 nt Set A1 or B1, 24 UDIs (-20 °C)

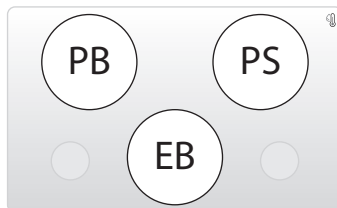


B)

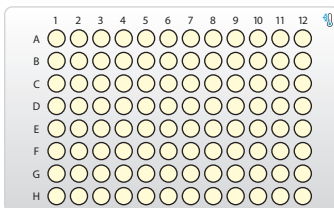
CORALL Library Generation Module, 96 reactions (-20 °C)



Purification Module 022.96 (+4 °C)



Lexogen UDI 12 nt Set A1, A2, A3, A4, or B1, 96 UDIs (-20 °C)



Library Amplification Module, 96 reactions (-20 °C)

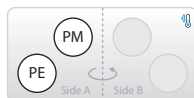


Figure 2. Location of kit components. All kits include Purification Modules and are supplied with Lexogen UDI 12 nt Sets. A) 24 prep kits contain either Set A1 or B1 with 24 UDIs. B) 96 prep kits contain either Set A1, A2, A3, A4, or B1 with 96 UDIs. The 384 prep kit contains 4 different UDI 12 nt plates (Sets A1-A4), and 4 of each modules.

Kit Component	Tube / Plate Label	Volume*			Storage
		24 preps	96 preps	384 preps	
Reverse Transcription Mix	RTM ●	476 µl	2x 951 µl	8x 951 µl	🧊 -20 °C
Reverse Transcription Mix Long	RTL ●	476 µl	2x 951 µl	8x 951 µl	🧊 -20 °C
Displacement Stop Primer	DSP ●	27 µl	106 µl	4x 106 µl	🧊 -20 °C
Enzyme Mix 1	E1 ●	27 µl	106 µl	4x 106 µl	🧊 -20 °C
Ligation Mix	LM ●	951 µl	3x 1,268 µl	12x 1,268 µl	🧊 -20 °C
Dithiothreitol	DTT ●	27 µl	106 µl	4x 106 µl	🧊 -20 °C
Linker Oligo	LO ●	27 µl	106 µl	4x 106 µl	🧊 -20 °C
Enzyme Mix 2	E2 ●	53 µl	212 µl	4x 212 µl	🧊 -20 °C
Bead Diluent	BD ○	2,086 µl	8,343 µl	4x 8,343 µl	🧊 -20 °C / 🌡 +4 °C
Library Amplification Module					
PCR Mix	PM ○	185 µl	740 µl	4x 740 µl	🧊 -20 °C
PCR Enzyme Mix	PE ○	27 µl	106 µl	4x 106 µl	🧊 -20 °C
Lexogen UDI 12 nt Sets					
Lexogen UDI 12 nt Set A1 or B1	UDI12A_0001-0024, UDI12B_0001-0024	10 µl / reaction			🧊 -20 °C
Lexogen UDI 12 nt Set A1, A2, A3, A4, or B1	UDI12A_0001-0096, UDI12A_0097-0192, UDI12A_0193-0288, UDI12A_0289-0384, UDI12B_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	1,307 µl	5,228 µl	4x 5,228 µl	🌡 +4 °C
Purification Solution	PS	1,901 µl	7,604 µl	4x 7,604 µl	🌡 +4 °C
Elution Buffer	EB	3,221 µl	12,884 µl	4x 12,884 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.

ATTENTION: Before starting this protocol, please read the [General Guidelines for Lexogen Kits](#), which are available online. These provide a detailed overview of RNA and kit component handling, as well as general RNA input requirements.

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. Please see Appendix A, p.27 for more information on RNA input and Appendix C, p.31 for information on library quantification and quality control.

4. Detailed Protocol - Short Insert Sizes (RTM)

ATTENTION: This protocol is designed to generate libraries with an average size of ~350 bp (average insert size: ~150 bp). For longer insert sizes, follow the protocol provided on p.18.

4.1 Optional Enrichment / Depletion

Prior to library preparation, samples can be either rRNA-depleted or poly(A) enriched. For depletion, we recommend RiboCop rRNA Depletion Kits (Cat. No. 125 - 127, 144, 145); bundles are available for RiboCop HMR V2 (Cat. No. 183 - 184) and HMR+Globin (Cat. No. 185 - 186). Poly(A) RNA Selection is included in the CORALL mRNA-Seq V2 Library Prep Kits (Cat. No. 177 - 182). Total RNA from 1 ng - 1 µg can be used as input for poly(A) selection or rRNA depletion. Follow the instructions in the respective User Guides before proceeding to CORALL library generation as described below. Total RNA input from 0.1 ng - 100 ng is also possible.

4.2 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, 1 min	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: 369.6 µl RTM (= 14 µl x 24 rxn x 1.1)
+ 26.4 µl DSP (= 1 µl x 24 rxn x 1.1)
resulting in a total of 396 µl.

NOTE: Protocol modifications are required while working with FFPE samples. Please refer to Appendix A, p.27 for more information.

REMARK: At this point we recommend placing the Purification Reagents (**PB**, **BD O**, and **EB**) for step 6 at room temperature to equilibrate for at least 30 minutes before use.


ATTENTION: The input range for CORALL RNA-Seq V2 is 1 ng - 1 µg total RNA for poly(A) selection or rRNA depletion.

- 1 Prepare a mastermix of 14 µl Reverse Transcription Mix (**RTM ●**) and 1 µl Displacement Stop Primer (**DSP ●**) per sample. Mix thoroughly and spin down briefly.
- 2 Add 15 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 25 µl. Mix thoroughly, quickly spin down. Incubate for 3 minutes at 94 °C, then 15 minutes at 16 °C.
- 3 Prepare a mastermix of 4 µl **RTM ●** and 1 µl of Enzyme Mix 1 (**E1 ●**) per sample.
- 4 Spin down samples from step 2 and add 5 µl of **RTM/E1**. Mix thoroughly and briefly spin down.
- 5 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.

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.

- 6 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 5. Mix thoroughly and incubate for 5 minutes at room temperature.
- 7 Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
- 8 Remove and discard the clear supernatant without removing the plate from the magnet. Do not disturb the beads!
- 9 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.
- 10 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.

-
- 11 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:** Do not let the beads dry too long!
-
- 12 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.
-
- 13 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 17 at room temperature to give it at least 30 minutes to equilibrate.

- 14 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 15.
-

- 15 Add 40 µl of the **LM** / **DTT** / **LO** / **E2** mastermix to the purified reverse transcription product from step 13. Mix thoroughly and spin down briefly.
-

- 16 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 O**, **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.

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

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

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

20 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.

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

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


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

24 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.

25 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.

26 Leave the plate in contact with the magnet and let the beads dry for 5 -10 minutes or until all ethanol has evaporated. **ATTENTION:** Do not let the beads dry too long!

27 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.

28 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 B, p.29.  Safe stopping point. After elution, libraries can be stored at -20 °C.

4.3 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 these kits.

Preparation

PCR		Purification (Cat. No. 022)
PM ○ Lexogen UDI 12 nt Sets (A1 - A4, or B1) PE ○	<div>- thawed at RT - thawed at RT - keep on ice or at -20 °C</div> <div>} spin down before opening!</div>	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	<div>95 °C, 60 sec 95 °C, 15 sec 60 °C, 15 sec 72 °C, 60 sec 72 °C, 6 min 10 °C, ∞</div> <div>} 11- 25x Endpoint cycle number as determined by qPCR (Cat. No. 020), see Appendix B, p.29.</div>	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 B, p.29.

- 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 **33** at room temperature to equilibrate for at least 30 minutes.

29

Prepare a mastermix containing 7 µl PCR Mix (**PM** ○) and 1 µl PCR Enzyme (**PE** ○) per reaction.

30

Add 8 µl of the **PM / PE** mastermix to 17 µl of the eluted library.

31

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!

32

Conduct 11 - 25 cycles of PCR (determine the required cycle number by qPCR) with the following program: Initial denaturation at 95 °C for 60 seconds, 11 - 25 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 60 seconds, and a final extension at 72 °C for 6 minutes, hold at 10 °C. 📌 Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The 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.

33

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.

34

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

35

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

36


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.

37

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.

38

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

- 39 Remove and discard the clear supernatant without removing the PCR plate from the magnet. Do not disturb the beads!
-
- 40 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.
-
- 41 Repeat this washing step once for a total of two washes. **ATTENTION:** Remove the supernatant completely.
-
- 42 Leave the plate in contact with the magnet and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Do not let the beads dry too long!
-
- 43 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.
-
- 44 Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
-
- 45 Transfer 17 μ l of the supernatant into a fresh PCR plate. Do not to transfer any beads.
-
- 46 At this point, the libraries are finished and ready for quality control (Appendix C, p.31), pooling (for multiplexing, see Appendix D, p.34), and cluster generation.  Safe stopping point. Libraries can be stored at -20 °C.
-

5. Short Procedure - Short Insert Sizes (RTM)

ATTENTION: Refer to the respective User Guides for Poly(A) selection or RiboCop rRNA Depletion prior to COR-ALL library prep. Spin down solutions before opening tubes or plates. Thaw RTM ● and LM ● on a thermal shaker at 30 °C and 1,250 rpm before use.

3.5 hrs

Library Generation

Reverse Transcription

- ☐ Prepare a mastermix of 14 µl **RTM** ● and 1 µl **DSP** ● per sample. Mix well.
- ☐ Add 15 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.
- ☐ Prepare a mastermix of 4 µl **RTM** and 1 µl **E1** ● per sample. Mix well.
- ☐ Add 5 µl **RTM / E1** and 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 or ThermoFisher, Cat. No. S7585).
- ☐ 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
72 °C, 1 min
10 °C, ∞. Calculate the optimal cycle number for Endpoint PCR (see Appendix B, p.29).

} **35x**
(see p.29)

Endpoint PCR

- ☐ Prepare a mastermix with 7 µl PCR Mix (**PM** ○) and 1 µl PCR Enzyme (**PE** ○) per reaction.
- ☐ Add 8 µl of the **PM / PE** mastermix to 17 µl of the eluted library.
- ☐ Add 10 µl of one Unique Dual Index Primer pair (UDI12A_0001-0384, or UDI12B_0001-0096) to each sample. **ATTENTION:** Reseal opened index wells after use! Use only one UDI / sample.
- ☐ PCR: 95 °C, 60 sec
95 °C, 15 sec
60 °C, 15 sec
72 °C, 60 sec
72 °C, 6 min
10 °C, ∞. Safe stopping point.

} **11 - 25x**
(see p.29)

ATTENTION: Increased cycle numbers may be required for low input / low quality / FFPE RNA (see Appendix A, p.27 and Appendix B, p.29)

Standard Input

FFPE RNA

Purification

- ☐ Add 31.5 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.

- ☐ For FFPE RNA: Add 42 µl **PB** per reaction, mix well, incubate 5 min at RT.

6. Detailed Protocol - Long Insert Sizes (RTL)

ATTENTION: This protocol is designed to generate libraries with an average size of ~550 bp (average insert size: >150 bp). **Do not use this protocol for low quality / FFPE samples. For low quality / FFPE samples, refer to Appendix A, p.27 and follow the protocol on p. 9.**

6.1 Optional Enrichment / Depletion

Prior to library preparation, samples can be either rRNA-depleted or poly(A) enriched. For depletion, we recommend RiboCop rRNA Depletion Kits (Cat. No. 125 - 127, 144, 145); bundles are available for RiboCop HMR V2 (Cat. No. 183 - 184) and HMR+Globin (Cat. No. 185 - 186). Poly(A) RNA Selection is included in the CORALL mRNA-Seq V2 Library Prep Kits (Cat. No. 177 - 182). Total RNA from 1 ng - 1 µg can be used as input for poly(A) selection or rRNA depletion. Follow the instructions in the respective User Guides before proceeding to CORALL library generation as described below. Total RNA input from 0.1 ng - 100 ng is also possible.

6.2 Library Generation

Preparation

Reverse Transcription	Ligation	Purification
DSP ● – thawed at RT RTL ● – 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, 1 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 Long (**RTL** ●) is viscous! Thaw completely on a Thermo-Mixer 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: 369.6 µl RTL (= 14 µl x 24 rxn x 1.1)
 + 26.4 µl DSP (= 1 µl x 24 rxn x 1.1)
 resulting in a total of 396 µl.

REMARK: At this point we recommend placing the Purification Reagents (**PB**, **PS**, and **EB**) for step 6 at room temperature to equilibrate for at least 30 minutes before use.


ATTENTION: The input range for CORALL RNA-Seq is 1 ng - 1 µg total RNA for poly(A) selection or rRNA depletion. **RTL is not recommended for low quality / FFPE samples. For low quality / FFPE samples, use RTM and follow the protocol on p. 9.**

- 1 Prepare a mastermix of 14 µl Reverse Transcription Mix Long (**RTL**) and 1 µl Displacement Stop Primer (**DSP**) per sample. Mix thoroughly and spin down briefly.
- 2 Add 15 µl of the **RTL / 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 25 µl. Mix thoroughly, quickly spin down. Incubate for 1 minute at 94 °C, then 15 minutes at 16 °C. **OPTIONAL:** Skip the 1 minute incubation step at 94 °C to increase the average library size to ~700 bp.
- 3 Prepare a mastermix of 4 µl **RTL** and 1 µl of Enzyme Mix 1 (**E1**) per sample.
- 4 Spin down samples from step 2 and add 5 µl of **RTL / E1**. Mix thoroughly and spin down.
- 5 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.

Purification

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

- 6 Prepare a mastermix of 9 µl Purification Beads (**PB**) and 6 µl Purification Solution (**PS**) per sample and add 15 µl of **PB / PS** mastermix to each reaction product from step 5. Mix thoroughly and incubate for 5 minutes at room temperature.
- 7 Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
- 8 Remove and discard the clear supernatant without removing the plate from the magnet. Do not disturb the beads!
- 9 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.
- 10 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.

-
- 11 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:** Do not let the beads dry too long!
-
- 12 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.
-
- 13 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** O, and **EB**) for step 17 at room temperature to give it at least 30 minutes to equilibrate.

14 Prepare a mastermix containing 36 µl of Ligation Mix (**LM** ●), 1 µl of Dithiothreitol (**DTT** ●), 1 µl of the Linker 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 15.

15 Add 40 µl of the **LM / DTT / LO / E2** mastermix to the purified reverse transcription product from step 13. Mix thoroughly and spin down briefly.

16 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 O**, **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.

17 Prepare a mastermix of 9 µl Purification Beads (**PB**) and 76 µl Bead Diluent (**BD O**) per sample and add 85 µl of **PB / BD** mastermix to the ligation reaction from step 16. Mix thoroughly and incubate for 5 minutes at room temperature.

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

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

20 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.

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

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


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

24 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.

25 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.

26 Leave the plate in contact with the magnet and let the beads dry for 5 -10 minutes or until all ethanol has evaporated. **ATTENTION:** Do not let the beads dry too long!

27 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.

28 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 B, p.29.  Safe stopping point. After elution, libraries can be stored at -20 °C.

6.3 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 these kits.

Preparation

PCR		Purification (Cat. No. 022)
PM ○ Lexogen UDI 12 nt Sets (A1 - A4, or B1) PE ○	<div>- thawed at RT - thawed at RT - keep on ice or at -20 °C</div> <div>} spin down before opening!</div>	<div>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</div>
Thermocycler	<div>95 °C, 60 sec 95 °C, 15 sec 60 °C, 15 sec 72 °C, 60 sec 72 °C, 6 min 10 °C, ∞</div> <div>} 11- 25x Endpoint cycle number as determined by qPCR (Cat. No. 020), see Appendix B, p.29.</div>	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 B, p.29.

- 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 33 at room temperature to equilibrate for at least 30 minutes.

- 29 Prepare a mastermix containing 7 µl PCR Mix (**PM** ○) and 1 µl PCR Enzyme (**PE** ○) per reaction.
- 30 Add 8 µl of the **PM / PE** mastermix to 17 µl of the eluted library.
- 31 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!
- 32 Conduct 11 - 25 cycles of PCR (determine the required cycle number by qPCR) with the following program: Initial denaturation at 95 °C for 60 seconds, 11 - 25 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 60 seconds, and a final extension at 72 °C for 6 minutes, hold at 10 °C.  Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The 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.

- 33 Add 31.5 µl of thoroughly resuspended Purification Beads (**PB**) to each reaction. Mix well, and incubate for 5 minutes at room temperature.
- 34 Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
- 35 Remove and discard the clear supernatant without removing the plate from the magnet. Do not disturb the beads!
- 36 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.
- 37 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.
- 38 Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
- 39 Remove and discard the clear supernatant without removing the PCR plate from the magnet. Do not disturb the beads!

40

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.

41

Repeat this washing step once for a total of two washes. **ATTENTION:** Remove the supernatant completely.

42

Leave the plate in contact with the magnet and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Do not let the beads dry too long!

43

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.

44

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

45

Transfer 17 µl of the supernatant into a fresh PCR plate. Do not to transfer any beads.

46

At this point, the libraries are finished and ready for quality control (Appendix C, p.31), pooling (for multiplexing see Appendix D, p.34), and cluster generation.

🔒 Safe stopping point. Libraries can be stored at -20 °C.

7. Short Procedure - Long Insert Sizes (RTL)

ATTENTION: Refer to the respective User Guides for Poly(A) selection or RiboCop rRNA Depletion prior to CORALL library prep. Spin down solutions before opening tubes or plates. Thaw RTL ● and LM ● on a thermal shaker at 30 °C and 1,250 rpm before use.

ATTENTION: Do not use RTL with low quality and FFPE RNA!

3.5 hrs

Library Generation

Reverse Transcription

- ☐ Prepare a mastermix of 14 µl RTL ● and 1 µl DSP ● per sample. Mix well.
- ☐ Add 15 µl of RTL / DSP mastermix to 10 µl RNA sample. Mix well.
- ☐ Incubate for 1 min at 94 °C, then 15 min at 16 °C.
- ☐ Prepare a mastermix of 4 µl RTL and 1 µl E1 ● per sample. Mix well.
- ☐ Add 5 µl RTL / E1 and 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 + 6 µl PS, 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 + 76 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 or ThermoFisher, Cat. No. S7585).
- ☐ 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	}	35x (see p.29)
65 °C, 20 sec		
72 °C, 30 sec		
72 °C, 1 min		
- 10 °C, ∞. Calculate the optimal cycle number for Endpoint PCR (see Appendix B, p.29).

Endpoint PCR

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

95 °C, 15 sec	}	11 - 25x (see p.29)	ATTENTION: Increased cycle numbers may be required for low input (see Appendix A, p.27 and Appendix B, p.29)
60 °C, 15 sec			
72 °C, 60 sec			
72 °C, 6 min			
- 10 °C, ∞. ⚡ Safe stopping point.

Purification

- ☐ Add 31.5 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.

8. Appendix A: RNA Input

The recommended input for CORALL RNA-Seq V2 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. DNase treatment is encouraged, particularly for low quality/FFPE samples to prevent gDNA contamination. Any DNase treatment should be performed with extreme care to minimize the potential impact on RNA quality and yield. High quality RNA-Seq data relies on high quality input RNA. RNA samples should be free of salts, metal ions, and organic solvents which can be carried over from RNA extraction as contaminants may have a negative impact on the efficiency of the protocol. Ribosomal RNAs (rRNAs) will consume the majority of sequencing reads unless these are removed before CORALL library generation. 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 (UHRR) 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 CORALL mRNA-Seq V2 Kits for poly(A) RNA-Seq with CORALL. 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. 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. Follow the instructions provided in the Poly(A) Selection Kit User Guide (157UG069) for mRNA enrichment prior to CORALL library preparation.

Ribo-Depletion

For rRNA depletion of human / mouse / rat RNA samples we recommend using Lexogen's RiboCop rRNA Depletion Kits for Human/Mouse/Rat or Human/Mouse/Rat Plus Globin (Cat. No. 144 or 145). RiboCop also removes the mitochondrial rRNA (mt-rRNA), which can otherwise account for more than 40 % of the reads. RiboCop rRNA Depletion Kits for Bacteria (Cat. No. 125 - 127) and Yeast (Cat. No. 190) are also available. For RiboCop rRNA depletion the recommended input range is 1 ng - 1 µg. CORALL is also compatible with other commercially available rRNA depletion kits. For eukaryotic samples, we recommend the use of depletion methods that also remove mt-rRNAs. Please contact support@lexogen.com for more information.

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 B, p.29 for more details). The CORALL library prep protocol was also tested using RNA from different species, including human, hamster, mini pig, mouse, rat, *Arabidopsis sp.*, and various bacterial species, including *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 RNA-Seq V2 library prep, and amplified with 11 - 12 PCR cycles.

FFPE Samples

Protocol modifications are recommended for library preparation from FFPE samples with CORALL. The following recommendations have been evaluated with as low as 25 ng total RNA input. **ATTENTION:** Always use **RTM** for FFPE samples! Sequencing is recommended with SR50-SR75.

- Perform DNase I treatment prior to rRNA depletion (without an intermediate cleanup step). Avoid heat-inactivation!
- In step **33** 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-Set 4 (Cat. No. 141) contains SIRV Isoform Mix E0, ERCC RNA Spike-in controls, and 15 Long SIRV transcripts ranging from 4 kb - 12 kb. 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. SIRV-Set 3 is recommended for CORALL library preps. For further questions, about SIRV usage and spike-in amounts, please contact support@lexogen.com.

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 B: cDNA Quantification by qPCR and Library Reamplification

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 end-point 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 Appendix C, p.31).

The PCR Add-on Kit for Illumina (Cat. No. 020) is required for the following qPCR assay protocol. The Reamplification Add-on Kit for Illumina (080.96) is required for re-amplification of under-cycled libraries, see below for details.

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 Thermo Fisher, Cat. No. S7585) is also needed and must be supplied by the user.

ATTENTION: Do not use the 12 nt UDIs with the PCR Mix (PCR ●) from the PCR Add-on Kit for Illumina (Cat. No. 020)! 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 28 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 μ l of the template (see Fig. 3).

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).

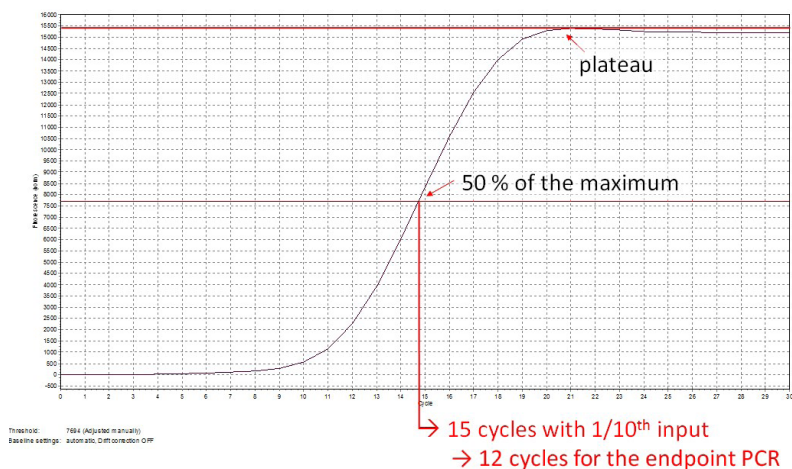


Figure 3. Calculation of the number of cycles for the endpoint PCR.

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.

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.

10. Appendix C: 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).

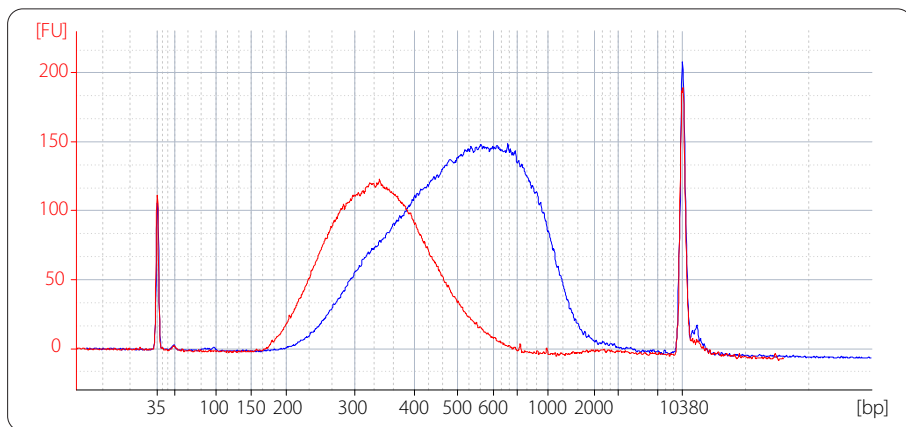


Figure 4. Bioanalyzer trace of typical CORALL libraries synthesized poly(A) enriched mRNA. 100 ng Universal Human Reference RNA (UHRR) was used with Lexogen's Poly(A) Selection Kit and the resulting poly(A) enriched RNA was inserted into CORALL library preparation using either RTM (red trace) or RTL (blue trace) and the respective protocol. Purified libraries were quality controlled using a Bioanalyzer High Sensitivity DNA assay. Libraries were amplified with 12 PCR cycles. The RTM synthesized library (red trace) has an average library size of 351 bp. The RTL synthesized library (blue trace) has an average library size of 572 bp, according to Bioanalyzer.

CORALL libraries generated with the RTL chemistry for longer insert sizes efficiently capture highly structured non-coding RNAs of ~300 nucleotides in size which are present in ribo-depleted RNA but not in poly(A) selected RNA. Examples include snoRNAs, snRNAs, or the RNA component of signal recognition particles (e.g., 7SL2). CORALL library prep protocols use a

fragmentation-free workflow, therefore the structure of these RNA species may lead to defined products in the final library. Inserts derived from these RNA species may therefore appear as distinct peaks in the library trace. Common RNA extraction and ribo-depletion protocols, such as RiboCop preserve RNA species < 300 nucleotides. When present in the input RNA (Fig. 5 A), peaks in the library trace directly correspond to these non-coding RNAs (Fig. 5 B). Figure 5 shows representative library traces generated from human reference RNA following ribo-depletion. Sequencing data quality is not affected by the distinct appearance of these RNAs in the libraries.

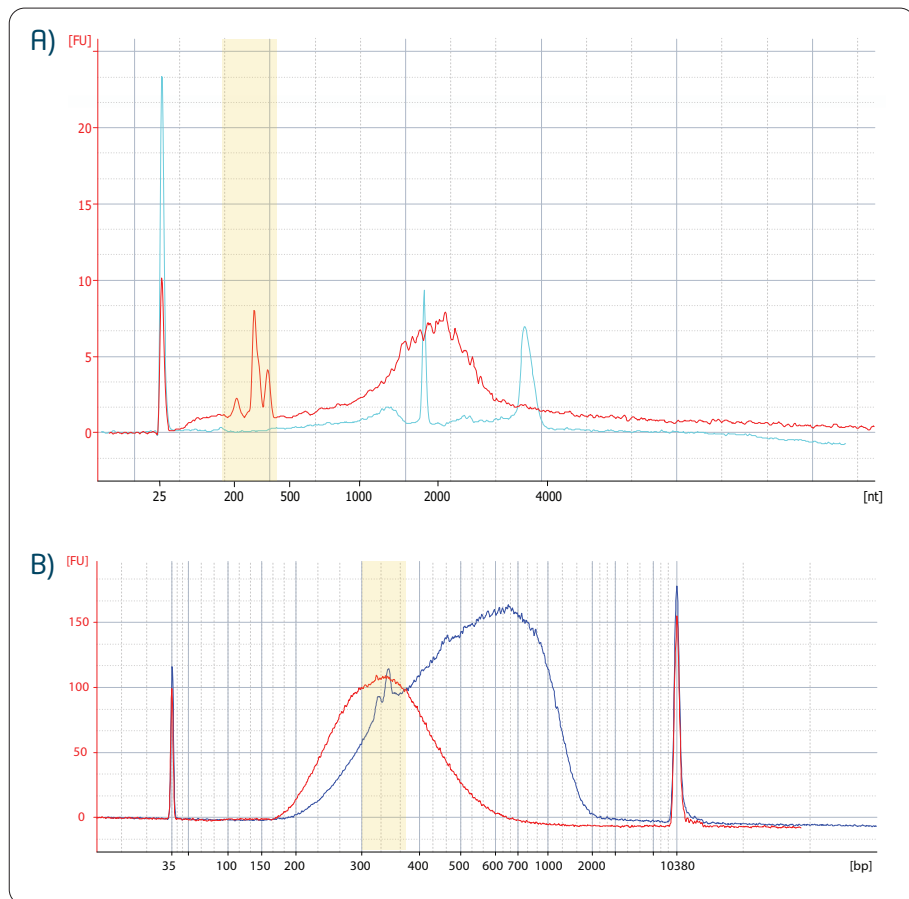


Figure 5. Bioanalyzer trace of ribo-depleted RNA and typical CORALL libraries generated from rRNA-depleted input RNA. Panel A: 500 ng Universal Human Reference RNA (UHRR) was used with Lexogen's RiboCop HMR V2 Kit and the resulting ribo-depleted RNA (red trace) is shown as overlay with 500 pg total RNA (cyan trace). Panel B: One fifth of the ribo-depleted RNA (corresponding to 100 ng total RNA) was inserted into CORALL. Either RTM (red trace) or RTL (blue trace) was used with the respective library preparation protocol. Purified libraries were quality controlled using a Bioanalyzer High Sensitivity DNA assay. Libraries were amplified with 12 PCR cycles. The RTM synthesized library (red trace) has an average library size of 355 bp. The RTL synthesized library (blue trace) has an average library size of 593 bp, according to Bioanalyzer.

Library Quantification Methods

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 μ l, adjust the total volume to 20 μ l 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 μ l of lane mix, add 45 μ l **PB**.



Follow the detailed protocol from step 34 onwards (p.14 - 15).

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 described in Appendix B, p.29) 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.

11. Appendix D: Multiplexing

Libraries prepared with the Lexogen UDI 12 nt Unique Dual Indexing Sets are suitable for sequencing on all Illumina instruments listed below. The Lexogen UDI 12 nt Sets enable adjustable read-out of 8, 10, or the full 12 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, flow cell type (paired-end, PE; single-read, SR), and chemistry, i5 indices are sequenced on the forward or the reverse complement strand:

Illumina Instruments	Flow Cell Type	Workflow	Lexogen UDI 12 nt Unique Dual Indexing
HiSeq 2000/2500 HiSeq 3000/4000	SR	Forward Strand (A)	Sets A1, A2, A3, and/or A4 (UDI12A_0001-0384) CORALL RNA-Seq V2: Cat. No. 171, 172, 173, 174, or 176 CORALL Total RNA-Seq V2 with RiboCop: Cat. No. 183 or 185 CORALL mRNA-Seq V2: Cat. No. 177, 178, 179, 180, or 182
HiSeq 2000/2500 MiSeq NovaSeq 6000 (v1.0 reagent kits)	PE		
iSeq 100 MiniSeq NextSeq 500 - 2000 HiSeq 3000/4000 NovaSeq 6000 (v1.5 reagent kits)	PE	Reverse Complement (B)	Set B1 (UDI12B_0001-0096) CORALL RNA-Seq V2: Cat. No. 175 CORALL Total RNA-Seq V2 with RiboCop: Cat. No. 184 or 186 CORALL mRNA-Seq V2: Cat. No. 181

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

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

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

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

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

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

Index Balance

In general, it is important that each nucleotide (A, C, G, and T) is present at each position of the index reads (Index 1 Read, i7; and Index 2 Read, i5), and that the signal intensity of each nucleotide is perfectly balanced to maintain optimal base calling accuracy and read quality. This is particularly critical for instruments that use two-channel detection (e.g., NextSeq, MiniSeq, and NovaSeq) and one-channel detection (iSeq). 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:

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

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.

12. Appendix E: 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 inserts generated with RTM are on average 150 bp long and libraries can be sequenced in single-read (SR) or paired-end (PE) formats. To minimize read overlap and trimming, optimal sequencing modes include SR 150 and PE 75. Longer inserts are generated when RTL is used. The differences in length distribution between RTM and RTL are shown in Fig. 4 on p.31 and in Fig. 5 on p.32. When RTL is used, longer read lengths can be applied (e.g., PE150). 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, the most convenient workflow for expression profiling analysis.

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, iSeq, and NovaSeq systems use PE flow cells only, which can also be used in single-read mode. Illumina defines Forward Strand (A) and Reverse Complement (B) Workflows for dual indexing read-out, which refer to the order of Index 2 read-out in relation to Read 2 Resynthesis. For more details about sequencing workflows, please refer to the [UDI 12 nt Unique Dual Indexing online Frequently Asked Questions \(FAQs\)](#), or contact support@lexogen.com. The example below shows the sequencing setup for dual-indexed CORALL libraries sequenced with the Reverse Complement Workflow (B) on a paired-end flow cell.

EXAMPLE: MiniSeq, iSeq, HiSeq 3000 / 4000 (PE), NovaSeq 6000 (v1.5 chemistry), and NextSeq instruments use the Reverse Complement Workflow (B) with the Multiplexing Index 2 (i5) Sequencing Primer. 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. 12 nt, 10 nt, or 8 nt can be read out optionally. If CORALL preps with UDI Set A (UDI12A_0001-0384) are sequenced on these machines, the i5 Index will be read out as reverse complement. **In this case, all 12 nt of the i5 Index must be read out if error correction shall be applied.**

CORALL libraries:

```
5'-(Read 1 Sequencing Primer)-3' UMI
5'AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N(12)-(Insert...
3'TTACTATGCCGTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N(12)-(Insert...
3'-(Index 2 (i5) Sequencing Primer)-5'

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

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. For more details please refer to the Instruction Manual for Lexogen 12 nt Unique Dual Indexing Add-on Kits (107UG223) or contact support@lexogen.com.

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):

Multiplexing Index 2 (i5) Sequencing Primer (not supplied):

5'AGATCGGAAGAGCGTCGTAGGGAAAGAGTGT 3'

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

5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

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

13. Appendix F: 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/docs/indexing.

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

Processing Raw Reads

We recommend the use of a general fastq quality control tool such as FastQC or NGS QC Toolkit to examine the quality of the sequencing run. These tools can also identify over-represented sequences, which may optionally be removed from the 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. The SIRV analysis pipeline SIRVsuite is available on github: <https://github.com/Lexogen-Tools>.

Data Analysis Solutions

An optimized CORALL Data Analysis Pipeline, which performs read quality control, mapping, Unique Molecular Identifier (UMI) deduplication, and transcript quantification, is available to download from our Lexogen Tools Github page ([GitHub - Lexogen-Tools/corall_analysis](https://github.com/Lexogen-Tools/corall_analysis)).

Additional web-based, automated data analysis solutions are available via the [Lexogen Data Analysis Solutions webpage](#). For further inquiries, please contact support@lexogen.com.

14. Appendix G: Revision History

Publication No. / Revision Date	Change	Page
171UG394V0101 Mar. 29, 2023	Amended information about analysis pipeline (BlueBee®).	39
171UG394V0100 Apr. 13, 2022	Initial Release.	

15. Appendix H: Notes

Associated Products:

020 (PCR Add-on Kit for Illumina)
022 (Purification Module with Magnetic Beads)
025, 050, 051, 141 (SIRVs Spike-in RNA Variant Control Mixes)
080 (Reamplification Add-on Kit for Illumina)
125 - 127, 144, 145, 190 (RiboCop rRNA Depletion Kit)
157 (Poly(A) RNA Selection Kit)

CORALL RNA-Seq Library Prep Kit V2 · User Guide

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