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FFPE Whole Transcriptome RNA-Seq Library Prep Kit with UDIs

User Guide

Catalog Numbers:
219-220 (CORALL FFPE Whole Transcriptome RNA-Seq Library Prep Kit with UDI 12 nt Set A1 or B1)
233-234 (RiboCop (HMR V2) and CORALL FFPE RNA-Seq Library Prep Kit with UDI 12 nt Set A1 or B1)
235 (DNA Removal Add-on)

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1. Overview

Lexogen's CORALL FFPE Whole Transcriptome RNA-Seq Kits enable 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 FFPE 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 FFPE RNA-Seq Library Prep Kits with UDIs include Lexogen UDI 12 nt Unique Dual Index Sets A1 or B1, or A1 - A4 for 384 reactions and RiboCop HMR V2 rRNA Depletion Kits for efficient removal of ribosomal RNA from FFPE samples. In addition, the DNA Removal Add-on can be seamlessly integrated upstream of RiboCop rRNA depletion without the need for additional intermediate purification steps. While total RNA without prior depletion or enrichment can also be used for CORALL FFPE library preparation, poly(A) selection is not recommended for FFPE RNA due to its fragmented nature. For FPPE RNA it is highly recommended to perform a DNase treatment to remove residual genomic DNA. Information regarding input RNA requirements can be found in Appendix A, p.22.

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

Lexogen'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 D, p.29). All purification steps are based on magnetic beads, rendering the protocol highly suitable for automation.

CORALL FFPE RNA-Seq 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. Conversion to other sequencing platforms is also possible following manufacturer's instructions. Data can be analyzed with a number of standard bioinformatic pipelines or free of charge through Lexogen's web-based data analysis platform Kangooroo. Information on read orientation, trimming, and Kangooroo is provided in Appendix E, p.31.

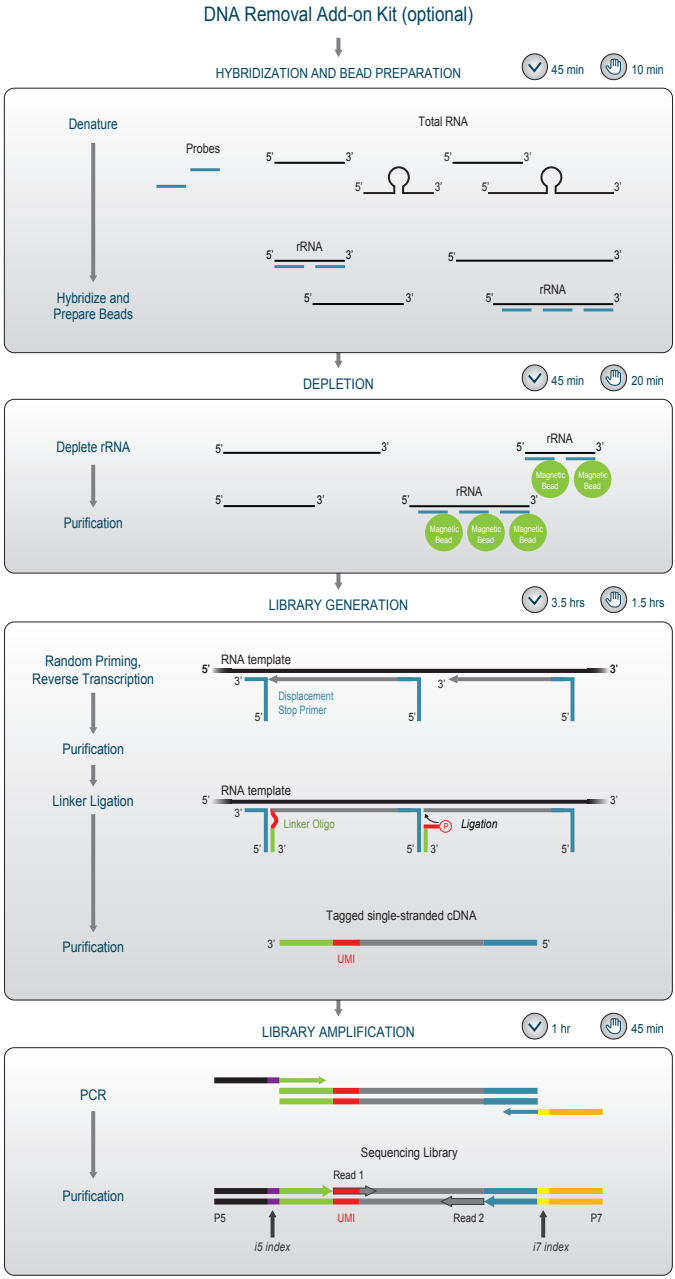


Figure 1. Schematic overview of the CORALL FFPE workflow. An optional DNA Removal Add-on Module can be seamlessly integrated upstream of RiboCop HMR V2 rRNA depletion. Depleted RNA can be directly channeled into library preparation.

2. Kit Components and Storage Conditions

Optional: DNA Removal Add-on Module

Kit Component	Tube Label	Volume*			Storage
		24 preps	96 preps	384 preps	
10x DNA Removal Buffer	DRB ●	27 µl	106 µl	4x 106 µl	-20 °C
DNA Removal Enzyme	DRE ●	27 µl	106 µl	4x 106 µl	-20 °C

*including ≥10 % surplus

RiboCop HMR V2 rRNA Depletion kit (Included in Cat. No. 233 and 234)

Kit Component	Tube Label	Volume*			Storage
		24 preps	96 preps	384 preps	
Hybridization Solution	HS ●	106 µl	423 µl	4x 423 µl	+4 °C
Probe Mix	HMR V2 ●	132 µl	528 µl	4x 528 µl	-20 °C / +4 °C
Depletion Beads	DB ●	1,980 µl	7,920 µl	4x 7,920 µl	+4 °C
Depletion Solution	DS ○	4,752 µl	19,008 µl	4x 19,008 µl	+4 °C
Purification Beads	PB ○	634 µl	2,535 µl	4x 2,535 µl	+4 °C
Purification Solution	PS ○	2,852 µl	11,405 µl	4x 11,405 µl	+4 °C
Elution Buffer	EB ○	317 µl	1,268 µl	4x 1,268 µl	-20 °C / +4 °C

*including ≥10 % surplus

Upon receiving the RiboCop kit, store all components at +4 °C. **EB** ○ and Probe Mix **HMR V2** ● can also be stored at -20 °C but avoid frequent freeze-thaw cycles. Before use, check the contents of **PS** ○ which may precipitate during shipping. If a precipitate is visible or the content appears milky, incubate at 37 °C until solution components dissolve completely. Equilibrate to room temperature again before use.

Corall FFPE RNA-Seq Library Preparation Kit (Included)

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
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	2x 1479 µl	-20 °C
PCR Enzyme Mix	**PE** ○	27 µl	106 µl	423 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 or B1	**UDI12A_0001-0096, 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**	1,584 µl	6,336 µl	4x 6,336 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 FFPE 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**).
- Lexogen PCR Add-on and Reamplification Kit V2 for Illumina (Cat. No. 208), for qPCR assay.
- 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.22 for more information on RNA input and Appendix B, p.24 for information on library quantification and quality control.

4. Detailed Protocol

ATTENTION: FFPE RNA is often highly fragmented. Corall FFPE is a fragmentation-free library prep, hence insert and library size therefore depended on the size distribution and quality of the input RNA.

4.1 Optional DNA Removal Add-on Module

Residual DNA should be removed from FFPE RNA prior to rRNA depletion. Most FFPE RNA extraction kits contain a DNase I treatment, however, this may not be complete depending on the amount of DNA that was co-purified. Lexogen offers a DNA Removal Add-on that can be seamlessly integrated upstream of RiboCop HMR V2 rRNA depletion. There is no need to purify samples between DNA removal and rRNA depletion. Total FFPE RNA from 5 ng - 1 µg can be used as input for DNA removal.

Preparation

DNA Removal
DRB ● – thawed at RT **DRE** ● – keep on ice or at -20 °C
Thermocycler 37 °C, 10 min

DNA Removal

Up to 8 µl total FFPE RNA, ranging from 5 ng - 1 µg can be inserted into the DNA removal step.
ATTENTION: For the preparation of mastermixes include a 10 % surplus per reaction.

EXAMPLE: Step 1 for 24 preps: 26.4 µl **DRB** ● (= 1 µl x 24 rxn x 1.1)
+ 26.4 µl **DRE** ● (= 1 µl x 24 rxn x 1.1)
resulting in a total of 52.8 µl.

1. Prepare a mastermix of 1 µl 10x DNA Removal Buffer (**DRB** ●) and 1 µl DNA Removal Enzyme (**DRE** ●) per sample. Mix thoroughly and spin down briefly.
2. Add 2 µl of the **DRB / DRE** mastermix to 8 µl of FFPE RNA sample. If a smaller volume of RNA is used, add RNase-free water to a total reaction volume of 10 µl. Mix thoroughly and quickly spin down.
3. Incubate for 10 minutes at 37 °C. ☞ Safe stopping point. After completing the reaction, the samples can be stored at -20 °C.
4. Add 16 µl RNase-free water to a total volume of 26 µl and insert the sample into step 2 of RiboCop rRNA depletion.

4.2 RiboCop HMR V2 rRNA Depletion

Prior to library preparation, FFPE samples should be rRNA-depleted. Total FFPE RNA from 5 ng - 1 µg can be used as input for rRNA depletion.

Preparation

Hybridization	Bead Preparation and Depletion	Purification
HS ● **HMR V2** ● } equilibrated at RT	**DB** ● **DS** ○ } equilibrated at RT	**PB** ○ – stored at +4 °C **PS** ○ – stored at +4 °C **80 % EtOH** – provided by user, **prepare fresh**! **EB** ○ – thawed at RT or stored at +4 °C
Thermomixer set to 75 °C / 1,250 rpm	Thermomixer set to 60 °C / 1,250 rpm	Equilibrate all reagents to room temperature for 30 minutes prior to use

Hybridization

The depletion Probe Mix (**HMR V2** ●) and total RNA are mixed, denatured, and hybridized.

ATTENTION: Important notes for RiboCop Hybridization Steps:

- If a thermomixer is not available, input amounts ≤500 ng of total RNA can be processed in PCR tubes or plates and incubated in a thermocycler, without shaking.
- Bead Preparation (p.11) can be performed during the incubation at step 5.
- Place the Purification Beads (PB ○) and Purification Solution (PS ○) for step 16 at room temperature prior to starting the protocol, to ensure these have at least 30 minutes to equilibrate.

1	Prepare 5 - 1,000 ng of total RNA in a total volume of 26 µl. Dilute using RNase-free water if required. If the DNA Removal Add-on was used prior to RiboCop rRNA depletion, add 16 µl RNase-free water to the DNA removal reaction and proceed to step 2.
2	Add 4 µl Hybridization Solution (**HS** ●).
3	Add 5 µl Probe Mix (**HMR V2** ●) and mix thoroughly until homogeneous.
4	Denature samples using a thermomixer at 75 °C for 5 minutes with agitation at 1,250 rpm.
5	Decrease the temperature of the thermomixer to 60 °C and incubate the samples for 30 minutes with agitation at 1,250 rpm. **OPTIONAL:** Transfer the samples from step 4 to a second thermomixer set to 60 °C.

Bead Preparation

Depletion Beads (**DB** ●) must be prepared before use. These steps may be performed as a batch for up to 6 samples.

ATTENTION: Mix Depletion Beads (**DB** ●) fully before use.

6	Transfer 75 µl Depletion Beads (**DB** ●) per reaction to a fresh tube.
7	Place the tube onto the magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear. Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
8	Add 75 µl Depletion Solution (**DS** ○) to the beads. Remove the tube from the magnet and resuspend the beads.
9	Place the tube back onto the magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear. Remove and discard the supernatant.
10	Repeat this washing step once (for a total of two washes).
11	Resuspend the beads in 30 µl Depletion Solution (**DS** ○).

Depletion

Depletion Beads (**DB** ●) are used to remove the probe-hybridized ribosomal RNA.

ATTENTION: Avoid the formation of air bubbles while mixing.

12	Spin down the hybridized sample from step 5 and add 30 µl of freshly prepared beads from step 11. Mix by pipetting up and down at least 8 times, or until homogeneous.
13	Put the sample back to the thermomixer and incubate at 60 °C for 15 minutes with agitation at 1,250 rpm.
14	Spin down briefly then place the sample on the magnet and let the beads collect for 5 minutes.
15	Recover and transfer 60 µl of the supernatant containing the rRNA-depleted RNA to a fresh tube. Avoid disturbing the collected beads to prevent rRNA contamination. **ATTENTION:** Do not throw away the supernatant.

Purification

The depleted RNA 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.

16	Add 24 µl Purification Beads (**PB** ○) and 108 µl Purification Solution (**PS** ○) to the supernatant. Mix well by pipetting. Incubate for 5 minutes at room temperature.
17	Place the sample onto a magnet and let the beads collect for 5 - 10 minutes or until the supernatant is completely clear. **NOTE:** The speed of bead collection depends on the strength of the magnet.
18	Remove and discard the clear supernatant without removing the sample from the magnet. Make sure that accumulated beads are not disturbed.
19	Add 120 µl of 80 % EtOH and incubate the beads for 30 seconds. Leave the sample on the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant. **ATTENTION:** When using 1.5 ml tubes use 150 µl of 80 % EtOH to ensure beads are fully covered by EtOH.
20	Repeat this washing step once for a total of two washes. Make sure the supernatant is removed completely.
21	Leave the sample in contact with the magnet and let the beads dry for 5 -10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting RNA recovery.
22	Add 12 µl of Elution Buffer (**EB** ○), remove the sample from the magnet and resuspend the beads properly in **EB** ○. Incubate for 2 minutes at room temperature.
23	Place the sample onto the magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
24	Transfer 10 µl of the supernatant into a fresh tube. Depleted RNA is now ready for CORALL FFPE library preparation. ☞ Safe stopping point. RNA can be stored at ≤-20 °C at this point.

4.3 CORALL FFPE 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 °C/ +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – stored at +4 °C
Thermocycler 94 °C, 3 min, 16 °C, 15 min; 25 °C, 10 min, 37 °C, 40 min, 42 °C, 10 min, 25 °C, 1 min	Thermocycler 37 °C, 30 min, 25 °C, 1min	Equilibrate all reagents to room temperature for 30 minutes prior to use.

Reverse Transcription

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

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

EXAMPLE: Step 1 for 24 preps: 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.

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

ATTENTION: The input range for CORALL FFPE RNA-Seq is 5 ng - 1 µg total RNA before rRNA depletion which amounts to approximately 150 pg - 30 ng of depleted RNA. Exceeding 100 ng rRNA depleted input is not recommended.

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** ○, 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** ○) 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** ○**, 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** ○) 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 (using Cat. No. 208), add an additional 2 µl of Elution Buffer (**EB**) to the eluted library. For further details please refer to 208UG591. ☞ Safe stopping point. After elution, libraries can be stored at -20 °C.

4.4 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** ○	- thawed at RT - thawed at RT - keep on ice or at -20 °C	**spin down before opening!**	**from standard library prep kits:** **PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – stored at +4 °C
Thermocycler	95 °C, 60 sec 95 °C, 15 sec 60 °C, 15 sec 72 °C, 60 sec 72 °C, 6 min 10 °C, ∞	11- 25x Endpoint cycle number as determined by qPCR (Cat. No. 208).	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 and Reamplification Kit V2 for Illumina (Cat. No. 208) is required.

- 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 (as determined by qPCR using Cat. No. 208) 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 42 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 B, p.24), pooling (for multiplexing, see Appendix C, p.27), and cluster generation. ☞ Safe stopping point. Libraries can be stored at -20 °C.

5. Short Procedures

ATTENTION: Spin down solutions before opening tubes or plates!

15 min DNA Removal (Optional)

	DNA Removal
☐	Prepare 5 - 1,000 ng total RNA in 8 µl.
☐	Prepare a mastermix of 1 µl **DRB** ● and 1 µl **DRE** ● per sample. Mix well.
☐	Add 2 µl **DRB / DRE** to 8 µl FFPE RNA, mix well.
☐	Incubate for 10 min at 37 °C ☞ Safe stopping point.
☐	Add 16 µl of RNase-free water to a total volume of 26 µl.

45 min Hybridization and Bead Preparation for rRNA Removal

	Hybridization
☐	Prepare 5 - 1,000 ng total FFPE RNA in 26 µl or directly use the output from the DNA Removal Add-on Module.
☐	Add 4 µl **HS** ●.
☐	Add 5 µl **HMR V2** ●, mix until homogeneous.
☐	Denature for 5 min at 75 °C / 1,250 rpm.
☐	Incubate for 30 min at 60 °C / 1,250 rpm.
	Bead Washing
☐	Resuspend **DB** ●, transfer 75 µl to a fresh tube.
☐	Place on magnet for 2 - 5 min, discard supernatant.
☐☐	Resuspend beads in 75 µl **DS** ○, incubate 2 min on magnet, discard supernatant. Repeat once.
☐	Resuspend beads in 30 µl **DS** ○.

45 min Depletion and Purification

	Depletion
☐	Spin down hybridized sample. Add 30 µl of prepared beads. Mix by pipetting 8x.
☐	Incubate for 15 min at 60 °C / 1,250 rpm. Spin down.
☐	Place on magnet for 5 min.
☐	Transfer 60 µl supernatant to a fresh tube. **ATTENTION:** The supernatant contains the rRNA depleted RNA.
	Purification
☐	Add 24 µl **PB** ○ and 108 µl **PS** ○, mix well, incubate for 5 min at RT.
☐	Place on magnet for 5 - 10 min, discard supernatant.
☐☐	Wash the beads twice with 120 - 150 µl 80 % EtOH, 30 sec. **NOTE:** Use 150 µl for 1.5 ml tubes.
☐	Air dry beads for 5 - 10 min. **ATTENTION:** do not over dry the beads!
☐	Add 12 µl **EB** ○, remove from magnet, mix well, incubate 2 min at RT.
☐	Place on magnet for 2 - 5 min, transfer 10 µl of the supernatant to a fresh tube. ☞ Safe stopping point.

ATTENTION: Spin down solutions before opening tubes or plates!

ATTENTION: Thaw RTM ● and LM ● on a thermal shaker at 30 °C and 1,250 rpm before use.

3.5 hrs Corall FFPE 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) Corall FFPE Library Amplification

qPCR [Strongly Recommended! Requires PCR Add-on and Reamplification Kit V2 (Cat. No. 208)]

- ☐ 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 **PM** ○, 5 µl **P5** ●, 5 µl **P7** ●, 1 µl **PE** ○, 1.4 µl of 2.5x SYBR Green I nucleic acid stain, and 13.9 µl of **EB**, per reaction. Mix well.
- ☐ PCR: 95 °C, 60 sec

  95 °C, 15 sec
  60 °C, 15 sec
  72 °C, 60 sec } **35x**

  72 °C, 6 min

  10 °C, ∞. Calculate the optimal cycle number for Endpoint PCR (please refer to 208UG591).

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 } **11 - 25x**

  72 °C, 6 min

  10 °C, ∞. ☞ Safe stopping point.

  ATTENTION: Increased cycle numbers may be required for low input and low quality FFPE RNA (see Appendix A, p.22)

Standard Input

Purification

- ☐ Add 42 µl **PB** per reaction, mix well, incubate 5 min at RT.
- ☐ Place on magnet for 2 - 5 min, discard supernatant.
- ☐ Add 30 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.
- ☐ Add 30 µl **PS**, mix well, incubate 5 min at RT.
- ☐ Place on magnet for 2 - 5 min, discard supernatant.
- ☐☐ Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.
- ☐ Air dry beads for 5 - 10 minutes. **ATTENTION:** Do not let the beads dry too long!
- ☐ Add 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.
- ☐ Place on magnet for 2 - 5 min, transfer 15 - 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.

6. Appendix A: RNA Input

The recommended input for the CORALL FFPE RNA-Seq Library Prep is ribosomal RNA-depleted (ribo-depleted) RNA. Total RNA without depletion or enrichment (100 pg to 100 ng) can also be used, if required. DNase treatment is essential for FFPE samples to prevent gDNA contamination. DNase treatment is often included in FFPE RNA extraction kits, but may be incomplete. SPLIT One-Step FFPE RNA Extraction is recommended for use with CORALL FFPE Library Preps. 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 FFPE library generation. Removal of rRNA can be achieved by rRNA depletion. Do not use poly(A) selection for FFPE RNA, as this will result in an extreme 3' bias due to the degraded nature of the sample. The amount of non-ribosomal RNA recovered depends on the method and the starting material. For most samples the non-ribosomal RNA fraction usually accounts for 1 % to 5 %. Higher recovery rates may indicate rRNA contamination.

DNA Removal Add-on Kit

Lexogen's DNA Removal Add-on Module can be used to remove DNA from FFPE samples. The recommended input range is 5 ng - 1 µg FFPE RNA. After DNA removal, the samples can be directly channeled into RiboCop HMR V2 rRNA depletion without an intermediate cleanup step. If other DNA removal protocols are used, we highly recommend adding an additional clean-up step before inserting the samples into RiboCop rRNA depletion. Deletion probes may be damaged if residual DNase activity is present, resulting in incomplete rRNA removal.

Ribo-Depletion

Corall FFPE RNA-Seq library prep includes Lexogen's RiboCop rRNA Depletion Kit for Human/Mouse/Rat (Cat. No. 144). RiboCop also removes the mitochondrial rRNA (mt-rRNA), which can otherwise account for more than 40 % of the reads. For RiboCop rRNA depletion the recommended input range is 5 ng - 1 µg FFPE RNA. CORALL is also compatible with other commercially available rRNA depletion kits, though 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 at least 25 - 50 ng of total FFPE RNA. **We strongly recommend performing a qPCR assay to determine the optimal PCR cycle number for your RNA input** (using Lexogen's PCR Add-on and Reamplification Kit V2 for Illumina, Cat. No. 208).

EXAMPLE: 50 ng of mouse liver total FFPE RNA inserted into DNA removal, RiboCop rRNA depletion and using 1.2 µl for CORALL FFPE RNA-Seq library prep required 14 PCR cycles. Note that cycle numbers may vary depending on the non-rRNA content of a tissue as well as the quality and integrity of the extracted FFPE RNA.

CORALL Libraries can be successfully generated from highly degraded FFPE RNA, down to a DV200 value of <10 % (see Fig. 3, p.25). The table below summarizes input recommendations and expected average library length for various FFPE RNA qualities.

FFPE RNA DV200	Recommended Total RNA Input	Min. Recommended Total RNA Input	Ave. Library Length*
>70 %	25 ng	5 ng	~330 bp
>50 %	50 ng	10 ng	~275 bp
> 30 %	100 ng	20 ng	~260 bp
<30 %	200 ng	50 ng	~250 bp

* Library length as evaluated on Agilent Bioanalyzer. Library length calculations vary between instruments and differ between Bioanalyzer, Fragment Analyer, TapeStation, and others. Libraries may appear shorter or longer on these instruments.

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 SIRVtranscripts 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 FFPE library preps. For FFPE RNA add SIRV-Set 3 (0.1 - 0.2 % of target RNA fraction) prior to DNase I treatment. 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.

7. Appendix B: Library Quality Control

Quality control of finished CORALL FFPE libraries is highly recommended and should be carried out prior to pooling and sequencing. A thorough quality control procedure should include the analysis of library concentration and size distribution (i.e., library shape).

Quality Control Methods

The analysis of a small volume of the amplified library with microcapillary electrophoresis has become standard practice for many NGS laboratories and generates information regarding library concentration and size distribution. Several electrophoresis platforms are available from various manufacturers. For low- to medium-throughput applications, we recommend the Bioanalyzer 2100 and High Sensitivity DNA chips (Agilent Technologies, Inc.). For high throughput applications instruments such as the Fragment Analyzer or 2200 TapeStation (Agilent Technologies, Inc.), or LabChip GX II (Perkin Elmer) are recommended. Typically, 1 µl of a CORALL library produced according to the directions in this manual is sufficient for analysis. Depending on the minimum sample loading requirements for each instrument, 1 µl of the finished library may be diluted to the required volume (e.g., 2 µl sample for TapeStation and 10 µl for LabChip GX II).

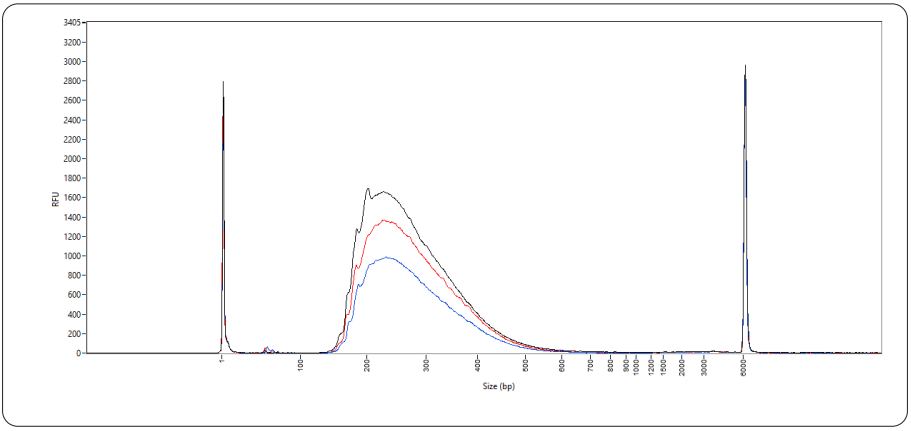


Figure 2. Fragment analyzer traces of typical CORALL FFPE libraries synthesized from *Mus musculus* (*Mm*) liver FFPE RNA (DV200 73 %, RQN1.2). 200 ng (red trace), 50 ng (blue trace) and 5 ng (black trace) FFPE RNA treated with the DNA Removal Add-on, then inserted into RiboCop rRNA depletion and a total RNA equivalent of 5 ng was used for CORALL FFPE library preparation. Libraries were amplified with 14 PCR cycles.

Due to the fragmented nature of FFPE RNA CORALL FFPE libraries contain smaller fragments, which may show up as distinct peaks (see black trace, peak at 199 bp). A peak at 199 bp contains an FFPE insert of 43 bp plus the UMI. Linker-linker artifacts without library insert typically run at 175 bp. To prevent wasting NGS reads on artifact peaks we recommend re-purification of the lanemix (see p. 26) in case there is a large peak at 175 bp.

Corall FFPE is a fragmentation-free library prep, hence the quality and degradation status of the input RNA determines the insert sizes that can be obtained.

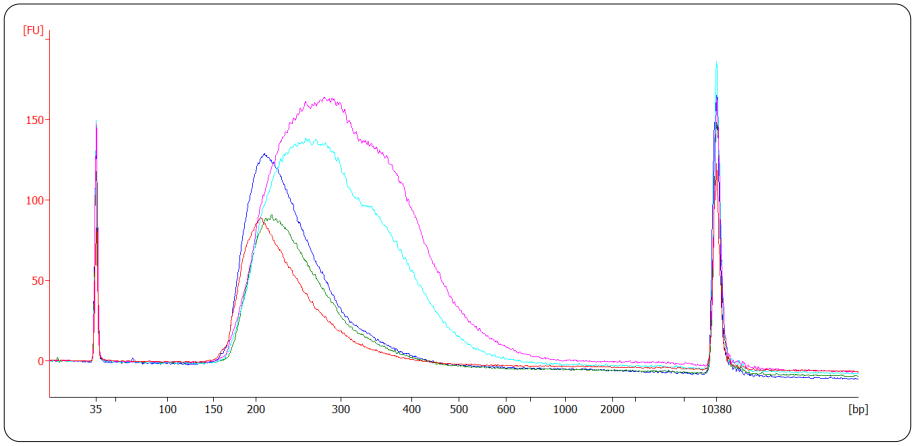


Figure 3. Bioanalyzer trace of ribo-depleted FFPE RNA of different quality inserted into CORALL FFPE library preparation. Input amounts listed refer to the total FFPE RNA inserted into DNA removal and RiboCop before the output is channeled into CORALL. Red trace: 50 ng Mm spleen FFPE RNA, DV200: 9 %, 15 cycles, average library size 256 bp. Blue trace: 20 ng human kidney tumor FFPE RNA, DV200: 45 % 13 cycles, average library size 260 bp Green trace: 20 ng human kidney normal FFPE RNA, DV200: 50 % 12 cycles, average library size 273 bp. Turquoise trace: 10 ng mouse liver FFPE RNA, DV200: 79 % 12 cycles, average library size 327 bp. Pink trace: 10 ng mouse liver fresh frozen tissue RNA, RIN9.1, 13 cycles, average library size 330 bp.

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, and should not compose more than 0 - 3 % of the total lane mix for sequencing. If the fraction of linker-linker, or other small fragments (≤175 bp) are too prominent, repurification of the lane mix prior to sequencing is advised.
Libraries or lane mixes can be repurified using the Purification Module with Magnetic Beads (Cat. No. 022) using the following protocol.

1. Measure the volume of the library or lane mix. If the volume is less than 20 µl, adjust the total volume to 20 µl using Elution Buffer (**EB**) or molecular biology-grade water (H_2O).
2. Add 1 volume (1x) of Purification Beads (**PB**). Mix thoroughly and incubate for 5 minutes at room temperature. **EXAMPLE:** For 50 µl of lane mix, add 50 µl **PB.**

Follow the detailed protocol from step 34 onwards (p.18).

Overcycling

A second peak in high molecular weight regions (between 1,000 - 9,000 bp) is an indication of overcycling. This could occur if cycle numbers are increased too much to compensate for lower input material. Prevent overcycling by using the qPCR assay as described in the PCR Add-on and Reamplification Kit V2 for Illumina User Guide (208UG591).

8. Appendix C: 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 and A1-A4** (UDI12A_0001-0384) CORALL FFPE Whole Transcriptome RNA-Seq: Cat. No. 219 RiboCop (HMR V2) and CORALL FFPE RNA-Seq: Cat. No. 233
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 FFPE Whole Transcriptome RNA-Seq: Cat. No. 220 RiboCop (HMR V2) and CORALL FFPE RNA-Seq: Cat. No. 234

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:

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

A template for molarity calculation is also available for download from www.lexogen.com.

After pooling the libraries, the prepared lane mix and any dilutions made for denaturing (e.g., 2 nM), should be reanalyzed to ensure the accuracy of the concentration. This can be performed according to steps 1 and 2 as above.

9. Appendix D: 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 FFPE inserts are on average 110 bp long and libraries, depending on the quality of the FFPE input RNA and can be sequenced in single-read (SR) or paired-end (PE) formats. To minimize read overlap and trimming, sequencing modes such as SR 100 or PE 75 are recommended. 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.

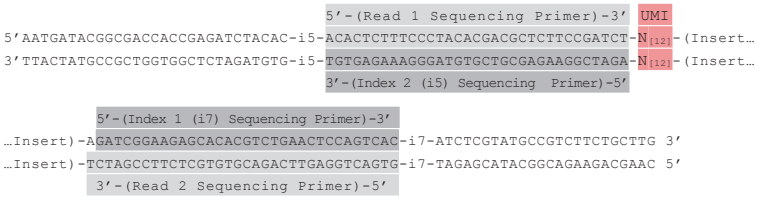
For information on loading amounts for the various sequencing instruments please refer to the CORALL online Frequently Asked Questions (FAQs), or contact support@lexogen.com.

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:



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 (198UG445) 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' AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT 3'

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied):
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

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

10. Appendix E: 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.
In addition 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 N_{12} Unique Molecular Identifiers (UMIs) at the start of Read 1. Hence, the first 12 nucleotides of Read 1 can be trimmed before proceeding to alignment. Alternatively, a less stringent aligner could be used with relaxed settings. Low quality sequences and adapter sequences should be trimmed. In case an adapter sequence is detected at the 3' end of Read 2, an additional 12 nucleotides upstream of the adapter can also be trimmed (i.e., the UMI sequence).

Alignment

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

Read Counting and Downstream Analyses

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

Free data analysis on Lexogen's web-based data analysis platform Kangooroo

With each purchased CORALL kit, you receive a voucher code for free data analysis on Kangooroo - Lexogen's web-based data analysis platform (kangooroo.com). The voucher code can be found on a label located on the cardboard above the microtube holder (or one of the holders, if the kit contains more than one). Each provided code allows for the same number of data analysis pipeline runs as the number of reactions included in the library prep kit, including differential expression (DE) analysis. The maximum amount of total data that can be uploaded is 10 GB for each sample, which is sufficient for most standard CORALL experiments.

The voucher code covers the download of the majority of the final results. If you are interested in downloading all data, including pre-processing data like trimmed FASTQ files or BAM files, or if you need to analyze bigger files, please contact us at sales@lexogen.com to purchase additional codes.

In addition, 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).

11. Appendix F: Revision History

Publication No. / Revision Date	Change	Page
219UG781V0100 Apr. 8, 2024	Initial Release.	

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Associated Products:

022 (Purification Module with Magnetic Beads)
025, 050, 051, 141 (SIRVs Spike-in RNA Variant Control Mixes)
144 (RiboCop rRNA Depletion Kit for Human/Mouse/Rat (HMR) V2)
208 (PCR Add-on Kit and Reamplification Kit V2 for Illumina)
236 (SPLIT One-Step FFPE RNA Extraction Kit)

CORALL FFPE Whole Transcriptome RNA-Seq Library Prep Kit · User Guide

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