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3' mRNA-Seq V2 Library Prep Kit REV with Unique Dual Indices (12nt)

User Guide

Catalog Numbers:
225 (QuantSeq 3' mRNA-Seq V2 Library Prep Kit REV with UDI Set B)

225UG675V0101

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Table of Contents

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1. Overview

Lexogen's QuantSeq 3' mRNA-Seq V2 Library Prep Kit Reverse (REV) with 12 nt Unique Dual Indices (UDIs) enables library preparation to generate Illumina-compatible libraries from polyadenylated RNA within 4.5 hours. The QuantSeq protocol generates only one fragment per transcript, resulting in extremely accurate gene expression values, and the sequences obtained are close to the 3' end of transcripts.

QuantSeq uses total RNA as input, hence no prior poly(A) enrichment or rRNA depletion is required. Information on input requirements can be found in Appendix A, p.18. Library generation is initiated by oligo(dT) priming (Fig. 1). The primer already contains a partial Illumina-compatible linker sequence. After first strand synthesis, the RNA is removed and second strand synthesis is initiated by random priming. The random primer also contains a partial Illumina-compatible linker sequence. No purification is required between first and second strand synthesis. The insert size is optimized for single-read lengths of 75 - 150 bases (SR75 - 150).

Second strand synthesis is followed by a magnetic bead-based purification step. The library is then amplified, introducing the sequences required for cluster generation. Lexogen's 12 nt Unique Dual Indices for multiplexing are included in the kit and are introduced during the PCR amplification step.

With QuantSeq Reverse, the Read 1 linker sequence is introduced by the oligo(dT) primer. Here, a Custom Sequencing Primer (**CSP ●** Version 5, included in the kit) is required for Read 1. The sequence generated during Read 1 corresponds to the cDNA. QuantSeq REV can be used for paired-end sequencing, ensuring the **CSP ●** is used for Read 1. With QuantSeq REV the exact 3' end is pinpointed in Read 1.

QuantSeq REV maintains strand specificity to allow mapping of reads to their corresponding strand on the genome, enabling the discovery and quantification of antisense transcripts and overlapping genes. The kits include magnetic beads for the purification steps and hence are compatible with automation.

Additional information regarding protocol modifications, quality control, add-on modules, multiplexing, and sequencing guidelines are found in Appendices B - G (p.19 - p.26).

Each purchased QuantSeq kit provides a code for free data analysis, including differential expression (DE) analysis on the web-based pipeline service (for more details, please read Appendix G, p.26.).

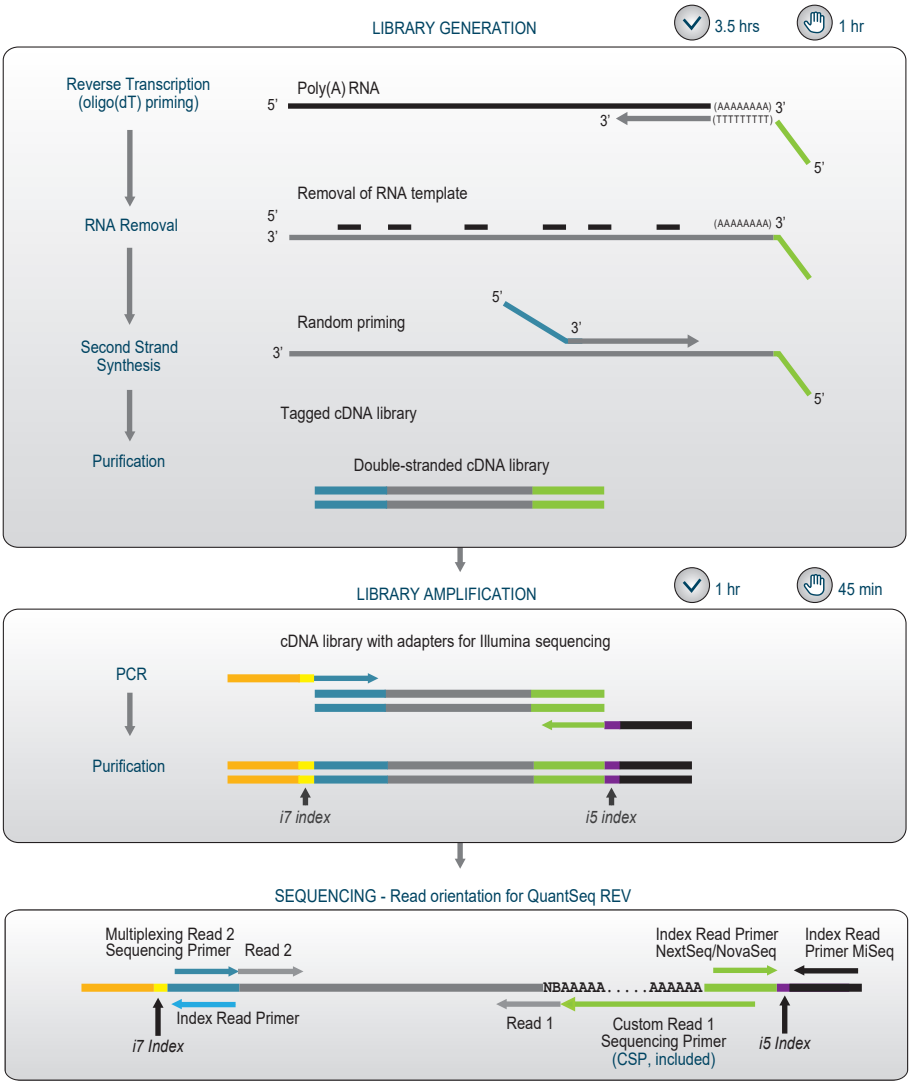


Figure 1. Schematic overview of the QuantSeq REV V2 library preparation workflow (Cat. No. 225). Sequencing read orientation for QuantSeq REV is depicted, with Read 1 (green) and Read 2 (blue). Read 1 reflects the cDNA sequence. QuantSeq REV is suitable for paired-end sequencing, and Read 2 reflects the mRNA sequence. A Custom Sequencing Primer (CSP Version 5, included in the kit) is required for Read 1.

2. Kit Components and Storage Conditions

Upon receiving the QuantSeq kit, store the Purification Module (Cat. No. 022) containing **PB**, **PS**, and **EB** at +4 °C, and the rest of the kit in a -20 °C freezer. **NOTE:** Before use, check the contents of **PS**. If a precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

Kit Component	Tube / Plate Label	Volume*		Storage
		24 preps	96 preps	
10x First Strand cDNA Synthesis Mix	**10xFS** ●	52.8 µl	211.2 µl	-20 °C
Oligo dT primer REV	**dT REV** ●	26.4 µl	105.6 µl	-20 °C
DTT	**DTT** ●	13.2 µl	52.8 µl	-20 °C
Enzyme Mix 1	**E1** ●	13.2 µl	52.8 µl	-20 °C
RNA Removal Solution	**RS** ○	132 µl	528 µl	-20 °C
Second Strand Synthesis Mix 1	**SS1** ●	264 µl	1,056 µl	-20 °C
Second Strand Synthesis Mix 2	**SS2** ●	105.6 µl	422.4 µl	-20 °C
Enzyme Mix 2	**E2** ●	26.4 µl	105.6 µl	-20 °C
Custom Sequencing Primer Version 5 (100 µM)	**CSP** ●	25 µl	50 µl	-20 °C
Library Amplification Module				
PCR Mix	**PM** ○	184.8 µl	739.2 µl	-20 °C
PCR Enzyme Mix	**PE** ○	26.4 µl	105.6 µl	-20 °C
Lexogen UDI 12 nt Sets				
Lexogen UDI 12 nt Set B1	**UDI12B_0001-0024**	10 µl / rxn		-20 °C
Lexogen UDI 12 nt Set B1	**UDI12B_0001-0096**		10 µl / rxn	-20 °C
Purification Module				
Purification Beads	**PB**	1,320 µl	5,280 µl	+4 °C
Purification Solution	**PS**	2,693 µl	10,772 µl	+4 °C
Elution Buffer	**EB**	2,904 µl	11,616 µl	+4 °C

*including ≥10 % surplus

Automation

QuantSeq is compatible with automation on various platforms. For further information see Appendix I, p.31, or contact us at support@lexogen.com.

ATTENTION: The Custom Sequencing Primer Version 5 (**CSP** ●) has to be provided to the sequencing facility together with the lane mix. For further details on the usage of the **CSP** ● see Appendix G, p.26. **Forward this information to your sequencing facility before starting a sequencing run.**

3. User-Supplied Consumables and Equipment

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

- 80 % fresh ethanol (**EtOH**, for washing of Purification Beads, **PB**).
- 10 mM Tris, pH 8.0 or RNase-free water.
- 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 assay.

Equipment

- Magnetic plate e.g., 96S Super Magnet Plate, article# A001322 from Alpaqua.
- Benchtop centrifuge (3,000 x g, rotor compatible with 96-well plates).
- Calibrated single-channel pipettes for handling 1 µl to 1,000 µl volumes.
- Calibrated multi-channel pipettes for handling 1 µl to 200 µl volumes.
- Thermocycler.
- UV-spectrophotometer to quantify RNA.
- Ice bath or ice box, ice pellets, benchtop cooler (-20 °C for enzymes).

Labware

- Ribonuclease-free low-binding pipette tips (aerosol barriers recommended).
- 1.5 ml reaction tubes, low-binding, certified ribonuclease-free.
- 200 µl PCR tubes or 96-well plates and caps or sealing foil.
- Vortex mixer.

Optional Equipment

- Automated microfluidic electrophoresis station (e.g., Agilent Technologies, Inc., 2100 Bioanalyzer).
- qPCR machine and library standards (for library quantification).
- Benchtop fluorometer and appropriate assays (for RNA quality control and library quantification).
- Agarose gels, dyes, and electrophoresis rig (for RNA quality control).

The complete set of material, reagents, and labware necessary for RNA extraction and quality control is not listed. Consult Appendix C, p.20, for more information on RNA quality. Consult Appendix E, p.22 for information on library quantification methods.

4. Detailed Protocol

4.1 Library Generation

Preparation

First Strand cDNA Synthesis	RNA Removal	Second Strand Synthesis	Purification
10xFS ● – thawed at RT **dT REV** ● – thawed at RT **DTT** ● – thawed at RT **E1** ● – keep on ice or at -20 °C	**RS** ○ – thawed at RT	**SS1** ● – thawed at 37 °C **SS2** ● – thawed at RT **E2** ● – keep on ice or at -20 °C	**PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – stored at +4 °C
85 °C, 3 min cool to 42 °C; 42 °C, 15 min	95 °C, 10 min cool to 25 °C	98 °C, 1 min, then cool to 25 °C (0.5 °C/sec) 25 °C, 30 min; 25 °C, 15 min	Equilibrate all reagents to room temperature for 30 minutes prior to use.

First Strand cDNA Synthesis - Reverse Transcription

An oligo(dT) primer containing an Illumina-compatible sequence at its 5' end is hybridized to the RNA and reverse transcription is performed.

ATTENTION: The minimum recommended input amount for QuantSeq REV V2 is 1 ng.

NOTE: Protocol modifications are recommended for low input (≤10 ng), low quality, and FFPE RNA samples. These are indicated as **"NOTE"** in the respective protocol steps (See also Appendix B, p.19, and Appendix C, p.20).

NOTE: If longer inserts are required, RNA + **dT REV** can be denatured separately and **10xFS** can be added to the mastermix prepared in step 3.
Longer library sizes may be beneficial for longer single-read sequencing, where increased length is beneficial for enhanced mapping rates.

1 Mix 1 ng - 500 ng of total RNA in a volume of up to 7 µl, with 1 µl oligo dT primer (**dT REV** ●) and 2 µl 10x First Strand cDNA Synthesis Mix (**10xFS** ●) in a PCR plate. If necessary (e.g., less than 7 µl input RNA was added), adjust the total volume to 10 µl with RNase-free water. Mix well by pipetting. Ensure the plate is tightly sealed, and spin down to collect the liquid at the bottom of the wells. **ATTENTION:** For low input / low quality / FFPE RNA, do not add **10xFS** ● to the RNA. Adjust the total volume to 10 µl.

OPTIONAL: Total RNA input volume can be increased to up to 16 µl for manual preps, and 12 µl for automated preps. See Appendix D, p.21 for more detailed information.

2 Denature the RNA / **dT REV / 10xFS** mix (or RNA / **dT REV** mix for low input / low quality / FFPE RNA) for 3 minutes at 85 °C in a thermocycler and then cool down to 42 °C. **ATTENTION:** Leave the reactions at 42 °C until step 4.

3 Prepare a mastermix containing 9 µl 10 mM Tris, pH 8.0 or RNase-free water, 0.5 µl DTT (**DTT** ●) and 0.5 µl Enzyme Mix 1 (**E1** ●) per reaction. Mix well, spin down, and pre-warm the mastermix for 2 - 3 minutes at 42 °C. **NOTE:** For low input / low quality / FFPE RNA, prepare a mastermix containing 2 µl **10xFS** ●, 7 µl 10 mM Tris, pH 8.0 or RNase-free water, 0.5 µl **DTT** ●, and 0.5 µl **E1** ● per sample. **ATTENTION:** Do not cool mastermixes on ice.

4 Quickly spin down the denatured RNA / **dT REV / 10xFS** samples from step 2 at room temperature to make sure all liquid is collected at the bottom of the wells. Place the samples back onto the thermocycler at 42 °C and carefully remove the sealing foil. Add 10 µl of the Tris (or H_2O) / **DTT** / **E1** mastermix to each reaction, mix well, and seal the plate. Spin down briefly and incubate the reactions for 15 minutes at 42 °C. **NOTE:** For low input / low quality / FFPE RNA, add 10 µl of the Tris (or H_2O) / **DTT** / **E1** / **10xFS** mastermix to each reaction. **ATTENTION:** Briefly spin down the samples and proceed immediately to step 5. Do not cool the samples below room temperature after reverse transcription.

RNA Removal

During this step the RNA template is degraded. This is essential for efficient second strand synthesis. Before removing the sealing foil after the first strand synthesis reaction, quickly spin down the plate to make sure all liquid is collected at the bottom of the wells.

OPTIONAL: At step 5, the Globin Block or BC1 Block Modules for QuantSeq can be used instead of the standard RNA Removal Solution (**RS** ○). Available modules include: RS-Globin Block, *Homo sapiens* (**RS-GBHs** ●, Cat. No. 070); RS-Globin Block, *Sus scrofa* (**RS-GBSs** ●, Cat. No. 071), or RS-BC1 Block, *Mus musculus* (**RS-BC1B** ●, Cat, No. 167). The use of Globin Block prevents the generation of amplifiable library fragments from globin mRNAs, which are present in blood total RNA, while BC1 Block prevents library fragment generation from BC1 RNA, which is highly abundant in mouse brain tissue. For further information on these modules please consult the respective User Guides: 070UG365 (Globin Block) and 167UG346 (BC1 Block).

5 Add 5 µl RNA Removal Solution (**RS** ○) directly to the first strand cDNA synthesis reaction. Mix well and reseal the plate using a fresh foil and spin down.

6 Incubate for 10 minutes at 95 °C, then cool down to 25 °C. Spin down and carefully remove the sealing foil. Proceed immediately to step 7.

Second Strand Synthesis

During this step the library is converted to dsDNA. Second strand synthesis is initiated by a random primer containing an Illumina-compatible linker sequence at its 5' end.

OPTIONAL: Instead of random primed Second Strand synthesis (SSS) a targeted Second Strand synthesis can be used. Please consult QuantSeq-Flex Second Strand Synthesis Protocol (028UG350). For targeted primed SSS using dT REV-generated cDNA please include an Illumina P7 sequence on the custom SSS primer (5' GACGTGTGCTCTTCCGATCT- target mRNA seq).

ATTENTION: **SS1** ● is a viscous solution. Thaw at 37 °C and mix thoroughly before use. If a precipitate is visible, incubate further at 37 °C, and mix until buffer components dissolve completely.

NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, **and EB**) for step 12 at room temperature to give it at least 30 minutes to equilibrate.

7	Add 10 µl Second Strand Synthesis Mix 1 (**SS1** ●) to the reaction. Mix well by pipetting, seal the plate, and spin down. **NOTE:** Use a pipette set to 30 µl for efficient mixing.
8	Incubate for 1 minute at 98 °C in a thermocycler, and slowly cool down to 25 °C at a reduced ramp speed of 0.5 °C/second. Incubate the reaction for 30 minutes at 25 °C. Quickly spin down the plate before removing the sealing foil.
9	Prepare a mastermix containing 4 µl Second Strand Synthesis Mix 2 (**SS2** ●) and 1 µl Enzyme Mix 2 (**E2** ●). Mix well. **ATTENTION:** Keep the mastermix at room temperature.
10	Add 5 µl of the **SS2** / **E2** mastermix per reaction. Mix well and spin down.
11	Incubate for 15 minutes at 25 °C, then briefly spin down. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The double-stranded library is purified using magnetic beads to remove all reaction components. The Purification Module (**PB**, **PS**, and **EB**) should equilibrate for 30 minutes at room temperature before use. The Purification Beads (**PB**) must be fully resuspended before use. Thorough mixing by pipetting or vortexing is recommended.

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

12	Add 16 µl of Purification Beads (**PB**) to each reaction. Mix well, and incubate for 5 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.
14	Remove and discard the clear supernatant without removing the PCR plate from the magnet. Do not disturb the beads.
15	Remove the plate from the magnet, add 40 µl of Elution Buffer (**EB**), and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
16	Add 56 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature. **NOTE:** For low input / low quality / FFPE RNA, add only 48 µl **PS** (see Appendix B, p.19 and Appendix C, p.20).
17	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
18	Remove and discard the clear supernatant without removing the plate from the magnet. Do not disturb the beads.
19	Add 120 µl of 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.
20	Repeat this washing step once for a total of two washes. Remove the supernatant completely, as traces of ethanol can inhibit subsequent PCR reactions.
21	Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.
22	Remove the plate from the magnet, add 20 µl of Elution Buffer (**EB**) per well, and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
23	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
24	Transfer 17 µl of the clear supernatant into a fresh PCR plate. Do not transfer any beads. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

4.2 Library Amplification

This section describes unique dual indexing PCR for multiplexing up to 384 libraries using the Lexogen 12 nt UDIs included in the QuantSeq 3′ mRNA-Seq REV V2 kit.

Preparation

PCR			Purification (Cat. No. 022)*	
PM ○ **PE** ○ **Lexogen UDI 12 nt Set B1**	- thawed at RT - keep on ice or at -20 °C - thawed at RT	**spin down before opening!**	**PB** **PS** **80 % EtOH** **EB**	– stored at +4 °C – stored at +4 °C – provided by user **prepare fresh!** – 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, ∞	12- 26x Endpoint cycle number as determined by qPCR (Cat. No. 208).	Equilibrate all reagents to room temperature for 30 min prior to use.	

PCR

The library is amplified to add the complete adapter sequences required for cluster generation and unique dual 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 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 with a fresh sealing foil after use to prevent cross contamination.
- Each well of the Lexogen UDI 12 nt Set is intended for single use only.

NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, and **EB**) for step 29 at room temperature to give it at least 30 minutes to equilibrate.

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

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

27 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!

28 Conduct 12 - 26 cycles of PCR (as determined by qPCR using Cat. No. 208) with the following program: Initial denaturation for 60 seconds at 95 °C, then 12 - 26 cycles of 15 seconds at 95 °C, 15 seconds at 60 °C and 60 seconds at 72 °C, and a final extension for 6 minutes at 72 °C, hold at 10 °C. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The finished library is purified from PCR components that can interfere with quantification. The Purification Module (**PB**, **PS**, and **EB**) should equilibrate for 30 minutes at room temperature before use. The Purification Beads (**PB**) must be fully resuspended before use. Thorough mixing by pipetting or vortexing is recommended.

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

29 For **QuantSeq** libraries **(standard input >10 ng**), add 35 µl of thoroughly resuspended Purification Beads (**PB**) to each reaction. **NOTE:** For **QuantSeq libraries generated from low input (≤10 ng) / low quality / FFPE RNA**, add only 31.5 µl **PB**. Mix well and incubate for 5 minutes at room temperature.

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

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

32 Remove the plate from the magnet, add 30 µl of Elution Buffer (**EB**), and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.

33 Add 30 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly 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 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.

37	Repeat this washing step once for a total of two washes. Remove the supernatant completely.
38	Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.
39	Remove the plate from the magnet, add 20 µl of Elution Buffer (**EB**) per well, and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
40	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
41	Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Do not transfer any beads. Libraries are now finished and ready for quality control (Appendix E, p.22), pooling (for multiplexing, Appendix F, p.24), and cluster generation. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

5. Short Procedure

ATTENTION: Spin down before opening tubes or plates!

3.3 hrs Library Generation

Standard Input	Low Input (≤10 ng) FFPE / Degraded RNA	Low Input (1 ng)
First Strand cDNA Synthesis		
☐ Mix 7 µl RNA, 1 µl **dT REV** ●, and 2 µl **10xFS** ●.	☐ Mix 9 µl RNA and 1 µl **dT REV** ●.	
☐ Incubate for 3 min at 85 °C, then cool to 42 °C. **Keep samples on thermocycler at 42 °C!**	☐ Incubate for 3 min at 85 °C, then cool to 42 °C. **Keep samples on thermocycler at 42 °C!**	
☐ Prepare a mastermix with 9 µl 10mM Tris, pH 8.0 or water, 0.5 µl **DTT** ● and 0.5 µl **E1** ● per reaction, mix well and pre-warm for 2 - 3 min at 42 °C.	☐ Prepare a mastermix with 7 µl 10 mM Tris, or water, 2 µl **10xFS** ●, 0.5 µl **DTT** ●, and 0.5 µl **E1** ● per reaction, mix well and pre-warm for 2 - 3 min at 42 °C.	
☐ Add 10 µl **Tris / DTT / E1** mix per reaction, mix well, and spin down. **Keep samples on thermocycler at 42 °C when adding mastermix!**	☐ Add 10 µl **Tris / 10xFS / DTT/ E1** mix per reaction, mix well, and spin down. **Keep samples on thermocycler at 42 °C when adding mastermix!**	
☐ Incubate for 15 min at 42 °C. **Proceed immediately to RNA Removal!**	☐ Incubate for 15 min at 42 °C. **Proceed immediately to RNA Removal!**	
RNA Removal		
☐ Add 5 µl **RS** ○, mix well.		
☐ Incubate 10 min at 95 °C, cool to 25 °C.		
Second Strand Synthesis		
☐ Add 10 µl **SS1** ●, mix well.		
☐ Incubate 1 min at 98 °C, slowly ramp down to 25 °C (0.5 °C/sec).		
☐ Incubate 30 min at 25 °C.		
☐ Prepare a mastermix with 4 µl **SS2** ● and 1 µl **E2** ● per reaction, mix well.		
☐ Add 5 µl **SS2 / E2** mix per reaction, mix well.		
☐ Incubate 15 min at 25 °C. ☞ Safe stopping point.		
Purification		
☐ Add 16 µl **PB** per reaction, mix well, incubate 5 min at RT.		
☐ Place on magnet for 2 - 5 min, discard supernatant.		
☐ Remove from magnet, add 40 µl **EB**, mix well, incubate 2 min at RT.		
☐ Add 56 µl **PS**, mix well, incubate 5 min at RT.	☐ For low input / low quality / FFPE: Add 48 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!		
☐ Remove from magnet, add 20 µl **EB**, 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.		

Standard Input	Low Input (≤10 ng and 1 ng) / Low Quality / FFPE

qPCR [Strongly Recommended! Requires PCR Add-on and Reamplification Kit V2 for Illumina (Cat. No. 208.96)]

- ☐ 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 **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 Mix (**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
  } **12 - 26x**

  72 °C, 6 min

  10 °C, ∞. ☞ Safe stopping point.

ATTENTION: Increased cycle numbers may be required for low input / low quality / FFPE RNA (see Appendix B, p.19 and Appendix C, p.20)

Standard Input	Low Input (≤10 ng and 1 ng) / Low Quality / FFPE
Purification	
☐ Add 35 µl **PB** per reaction, mix well, incubate 5 min at RT.	☐ For low input / low quality / FFPE: Add 31.5 µl **PB** per reaction, mix well, incubate 5 min at RT.
☐ Place on magnet for 2 - 5 min, discard supernatant.	
☐ Remove from magnet, add 30 µl **EB**, 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!	
☐ Remove from magnet, add 20 µl **EB**, 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 and PCR Cycles

Total RNA is the intended input for QuantSeq REV. No prior rRNA depletion or poly(A) enrichment is required. As QuantSeq is a 3' mRNA-Seq protocol, both high and low-quality RNA can be used as input. Any total RNA sample that contains polyadenylated mRNA can be used, including e.g., bacterial RNA samples that have been previously polyadenylated.
QuantSeq has been tested extensively with high quality Universal Human Reference RNA (UHRR) across a wide range of input amounts (1ng - 500 ng).

Input Guidelines

- We recommend performing the protocol initially with 500 ng total RNA. RNA inputs ≥200 ng are recommended to detect low abundant transcripts efficiently.
- The recommended input range of high-quality total RNA is 1 ng - 500 ng for QuantSeq REV.
- Lower RNA inputs (≤10 ng), and low-quality RNA samples (including FFPE) require protocol modifications, including adjusting the number of PCR cycles for the endpoint PCR (see Appendix B, p.19 and Appendix C, p.20).
- The minimum recommended input for QuantSeq REV V2 libraries prepared from whole blood total RNA using the Globin Block Modules (RS-GB) is 50 ng. For BC1 Block Module (RS-BC1B), the minimum input is 10 ng of total RNA from mouse brain total RNA. For lower input amounts, mapping rates and gene detection may be reduced.
- **The optimal cycle number for your specific sample type should be determined using the qPCR assay** (using Lexogen's PCR Add-on and Reamplification Kit V2 for Illumina, Cat. No. 208). Libraries prepared with Globin or BC1 Block typically require one cycle more than libraries prepared from total RNA without Globin or BC1 Block.
- The number of PCR cycles optimal for a given input amount of total RNA can vary by up to four and should be determined for different sample types using the qPCR assay. The table below is provided as a reference only! Optimal cycle numbers could exceed these ranges depending on the sample type (e.g., species, tissue, RNA quality (e.g., FFPE RNA)).

Total RNA Input Amount	No. Cycles for Endpoint PCR**
1 ng*	22 - 26
10 ng*	18 - 21
100 ng	15 - 18
500 ng	12 - 15

* Using low input protocol modifications (See Appendix B, p.19).
** These values are provided as a **reference only!** Sample type influences the optimal cycle number, which should be determined by a qPCR assay (please refer to 208UG591).

7. Appendix B: Low Input RNA

Protocol modifications apply when using low input RNA (≤10 ng), low quality or degraded RNA, or RNA isolated from formalin-fixed, paraffin-embedded (FFPE) sample. These are shown in the table below. See also Appendix C, p.20 for specific information on protocol recommendations for FFPE samples.

Working with input amounts of 1 ng total RNA is challenging. For these very low input amounts, the duration of RNA removal is reduced to 5 minutes at step 29. Performing the qPCR assay to optimize the number of endpoint PCR cycles is required and we strongly recommend including a no-input control for library preparation.

Protocol Step	Standard Input (>10 ng)	Low Input (≤10 ng) FFPE / Degraded RNA	Low Input (1 ng)
Step 1	Add **dT REV**● and **10xFS**● to RNA samples. Do not place samples back on ice !	Add **dT REV**● only to RNA samples. Do not place samples back on ice !	
Step 3	Prepare Tris / **DTT/ E1** mastermix – pre-warm for 2 - 3 minutes at 42 °C.	Prepare Tris **/ 10xFS /DTT / E1** mastermix – pre-warm for 2 - 3 minutes at 42 °C.	
Step 4	Add pre-warmed mastermix to **RNA / 10xFS / dT REV** samples on the thermocycler at 42 °C. Incubate for 15 minutes at 42 °C.	Add pre-warmed mastermix to **RNA / dT REV** samples at room temperature on the thermocycler at 42 °C. Incubate for 15 minutes at 42 °C.	
Step 16	Add 56 µl of Purification Solution (**PS**).	Reduce volume of Purification Solution (**PS**) to 48 µl.	
Step 24	The qPCR assay is strongly recommended for optimizing the number of PCR cycles required for library amplification. This will prevent under- or overcycling of the libraries. The qPCR assay should be performed also when RNA samples are of: • Variable input amount • Variable quality (RIN / RQN) or purity (absorbance ratios: 260 / 280 and 260 / 230) • Variable type (e.g., species, tissue, cell type) • FFPE origin, or highly degraded		
Step 29	Add 35 µl of Purification Beads (**PB**) for dual-indexed libraries.	Reduce volume of Purification Beads (**PB**) to 31.5 µl for dual-indexed libraries.	

8. Appendix C: Low-quality RNA - FFPE

RNA isolated from formalin-fixed, paraffin embedded (FFPE) samples is often heavily degraded. As QuantSeq is a 3' mRNA-Seq protocol, it is highly suitable for FFPE RNA.
For FFPE samples only minor protocol adjustments are required, specifically:

- Steps 1 and 2 : do not add **10xFS** and do not denature at 85°C in **10xFS** buffer.
- Reducing the volume of **PS** in step 16 to 48 µl.
- Reducing the volume of **PB** in step 29 to 31.5 µl for dual indexing PCR.

As the RNA amount is often a limiting factor with FFPE samples, QuantSeq was tested with 1 ng - 50 ng FFPE or degraded RNA input, including mouse (Mm) brain FFPE RNA input with a RIN of 1.8 (DV_{200} of 51 %). The DV_{200} is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV_{200}, the more degraded the RNA is.

ATTENTION:

- FFPE RNA samples are highly variable. Samples with lower mRNA content, or lower DV_{200} values may require more PCR cycles. **We strongly recommend performing a qPCR assay** using Lexogen's PCR Add-on and Reamplification Kit V2 for Illumina (Cat. No. 208) to determine the optimal cycle number for library amplification.
- FFPE RNA is highly degraded, hence the insert sizes are smaller than for non-degraded RNA samples (see also Appendix E, p.22). Keep this in mind when choosing your sequencing length.
- If you see that your FFPE RNA generates ~175 bp linker-linker products despite the above-mentioned protocol changes, repurification of the lane mix with 0.9x **PB** (e.g., 50 µl lane mix plus 45 µl of **PB**, incubating 5 minutes at room temperature, and following the protocol from step 30 on again) may be necessary.
- FFPE RNA can be contaminated with fragmented DNA, which may result in an overestimation of inserted RNA and/or in a high number intronic and intergenic reads in NGS samples. For FFPE RNA it is recommended to perform a DNase I treatment, or to distinguish between RNA and DNA when quantifying your input material. Heat inactivation of DNase I should be avoided, and the enzyme should be deactivated by other means such as phenol / chloroform extraction or silica column purification.
- Mitochondrial ribosomal RNAs (mt-rRNAs) are polyadenylated and hence will also be reverse transcribed and converted into a cDNA library if oligo(dT) priming is used during First Strand Synthesis. mt-rRNAs can therefore be observed, but will only represent a minor fraction of the reads when using a 3' mRNA Seq protocol.

- Optionally, an rRNA depletion method, which also removes mt-rRNAs, such as Lexogen's Ribo-Cop rRNA Depletion Kit (144.24 and 144.96) can be used before starting the QuantSeq library preparation.
- If adding Spike-in controls (e.g., SIRV-Set 3, Cat. No. 051), these should be added prior to any DNase I treatment. For further questions, please contact support@lexogen.com.

9. Appendix D: High volume RNA Input

QuantSeq REV V2 is compatible with larger RNA input volumes than 7 µl (standard protocol described on p. 8).

Total RNA input volume can be increased to up to 16 µl for manual preparations, as described below. **OPTIONAL:** For automation we recommend having at least 5 µl of Tris / **DTT** / **E1** mastermix to pipette in step 4, hence a maximum of 12 µl of input RNA.

1	Mix 1 ng - 500 ng of total RNA in a volume of up to 16 µl, with 1 µl oligo dT primer (**dT REV** ●) and 2 µl 10x First Strand cDNA Synthesis Mix (**10xFS** ●) in a PCR plate. Mix well by pipetting. Ensure the plate is tightly sealed, and spin down to collect the liquid at the bottom of the wells.
2	Denature the RNA / **dT REV / 10xFS** mix for 3 minutes at 85 °C in a thermocycler and then cool down to 42 °C. **ATTENTION:** Leave the reactions at 42 °C until step 4.
3	Prepare a mastermix containing 0.5 µl DTT (**DTT** ●) and 0.5 µl Enzyme Mix 1 (**E1** ●) per reaction. Mix well, spin down, and pre-warm the mastermix for 2 - 3 minutes at 42 °C. **NOTE:** If you added less than 16 µl RNA in step 1, complete the **DTT / E1** mastermix with 10 mM Tris, pH 8.0 or RNase-free water so that the final volume of Tris / **DTT / E1 / dT REV / 10xFS** / RNA will reach 20 µl. Pre-warm the Tris / **DTT / E1** mastermix for 2 - 3 minutes at 42 °C. **ATTENTION:** Do not cool mastermixes on ice.
4	Quickly spin down the denatured RNA / **dT REV / 10xFS** samples from step 2 at room temperature to make sure all liquid is collected at the bottom of the wells. Place the samples back onto the thermocycler at 42 °C and carefully remove the sealing foil. Add the (Tris) / **DTT** / **E1** mastermix to each reaction, mix well, and seal the plate. Spin down briefly and incubate the reactions for 15 minutes at 42 °C. **ATTENTION:** After the reverse transcription, briefly spin down the samples and proceed immediately to step 5. Do not cool the samples below room temperature after reverse transcription.

10. Appendix E: Library Quality Control

Quality control of finished QuantSeq 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 QuantSeq library produced according to the directions in this manual is sufficient for analysis. Depending on the minimum sample loading requirements for each instrument, 1 µl of the finished library may be diluted to the required volume (e.g., 2 µl sample for TapeStation and 10 µl for LabChip GX II).

More accurate library quantification can be achieved with custom or commercially available qPCR assays. With these assays, the relative or absolute abundance of amplifiable fragments contained in a finished QuantSeq 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.

Typical Results

QuantSeq libraries are intended for a high degree of multiplexing, and hence libraries do not need to be extensively amplified. Library yield, shape, and average insert size may vary depending on the type of input sample, see Figures 2 and 3. Degraded or FFPE samples typically produce shorter libraries than high quality Universal Human Reference RNA (UHRR). The majority of inserts are greater than 75 bp in size, corresponding to final library fragment sizes ≥200 bp.

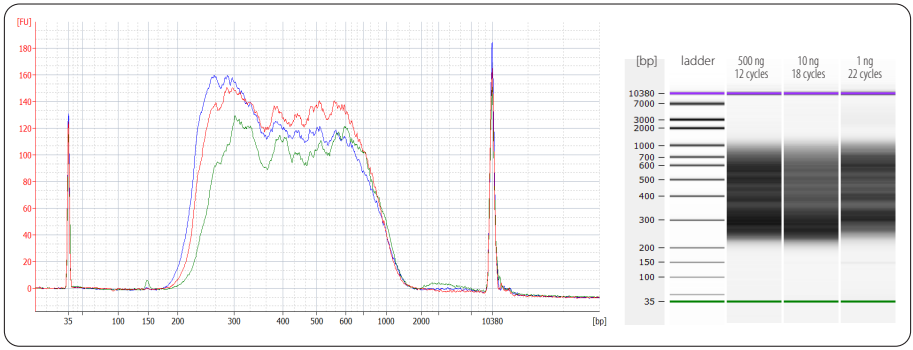


Figure 2. Bioanalyzer traces of QuantSeq REV V2 libraries prepared from different input amounts of total RNA input (UHRR). Libraries were prepared with the standard protocol, using 500 ng (red trace, 12 PCR cycles) and with 10 ng (blue trace, 18 PCR cycles) . Libraries with 1 ng (green trace, 22 PCR cycles) of UHRR input were prepared using low input protocol modifications (see Appendix B, p.19). Endpoint PCR was performed using the unique dual indices.

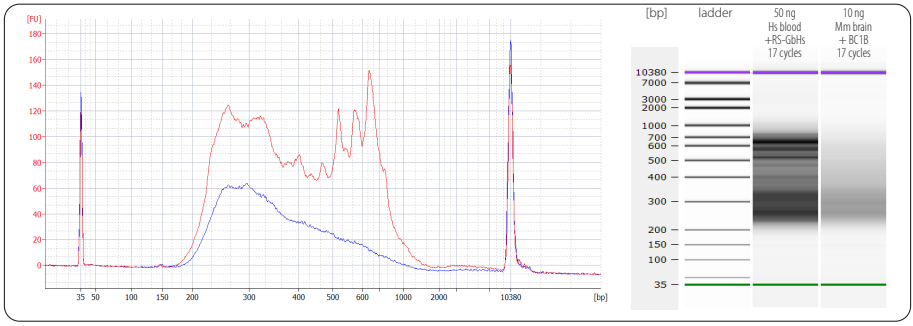


Figure 3. Bioanalyzer traces of QuantSeq REV V2 libraries synthesized from 50 ng human whole blood RNA (red trace) including RS-GBHs blocker (Cat. No. 70) or 10 ng (dark blue trace) mouse (Mm) brain RNA using RS-BC1B blocker (Cat. No. 167).

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).

11. Appendix F: Multiplexing

Libraries prepared with the Lexogen UDI 12 nt Unique Dual Indexing Sets are suitable for sequencing on the 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 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)	Lexogen UDI 12 nt **Sets A1, A2, A3, and/or A4** (UDI12A_0001-0384) Cat. No. 101 - 104.96 or 156.384
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) NovaSeq X	PE	Reverse Complement (B)	Lexogen UDI 12 nt **Set B1** (UDI12B_0001-0096) Cat. No. 105.96 QuantSeq 3' mRNA-Seq V2 REV: Cat. No. 225

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 QuantSeq REV 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.

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 180 - 2,000 bp to include the whole library size distribution, and to exclude any linker-linker artifacts (175 bp), or overcycling bumps (>2,000 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.

Lane Mix Repurification to Remove Linker-Linker Artifacts

A shorter side-product representing linker-linker artifacts is sometimes visible at ~175 bp (for dual indexed libraries), and should not compose more than 0 - 3 % of the total lane mix for sequencing. If the fraction of linker-linker, or other small fragments (≤175 bp) are too prominent, repurification of the lane mix prior to sequencing is advised.

Libraries or lane mixes can be repurified using the Purification Module with Magnetic Beads (Cat. No. 022) using the following protocol:

1. Measure the volume of the library or lane mix. If the volume is less than 20 µl, adjust the total volume to 20 µl using Elution Buffer (**EB**) or molecular biology-grade water (H_2O).
2. Add 0.9 volumes (0.9x) of Purification Beads (**PB**). Mix thoroughly and incubate for 5 minutes at room temperature. **EXAMPLE:** For 50 µl of lane mix, add 45 µl **PB.**

Follow the detailed protocol from step 30 onwards (p.13).

12. Appendix G: Sequencing*

General

The amount of library loaded onto the flow cell will greatly influence the number of clusters generated. Machine-specific loading instructions can be found in our QuantSeq online Frequently Asked Questions (FAQs).

Multiplexing with Other Library Types

We do not recommend multiplexing Lexogen libraries with libraries from other vendors in the same sequencing lane.
Though this is possible in principle, specific optimization of index combinations, library pooling conditions, and loading amounts may be required, even for advanced users. Sequencing complex pools that include different library types at different lane shares may have unpredictable effects on sequencing run metrics, read quality, read outputs, and/or demultiplexing performance. Lexogen assumes no responsibility for the altered performance of Lexogen libraries sequenced in combination with external library types in the same lane (or run).

Due to size differences, libraries prepared with the Lexogen Small RNA-Seq Library Prep Kit (or any other small RNA library prep kit) should not be sequenced together with QuantSeq libraries. Please refer to the sequencing guidelines for each library type (library adapter details, loading amounts to use, and use of custom sequencing primers, etc.), which are provided in our Library Prep Kit User Guides, and online FAQs.

Dual Indexed Library Sequencing Workflows

The workflow for dual indexed library sequencing differs, depending on the Illumina instrument and flow cell type. Dual indexing can be performed on single-read (SR) and paired-end (PE) flow cells. All HiSeq systems support SR and PE flow cells. NextSeq, MiniSeq, MiSeq, and NovaSeq systems use PE flow cells only, which can also be used in single-read mode. Illumina defines 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 FAQs, or contact support@lexogen.com.

The example below shows the sequencing setup for dual indexed QuantSeq REV V2 libraries sequenced with the Reverse Complement Workflow (B) on a paired-end flow cell.

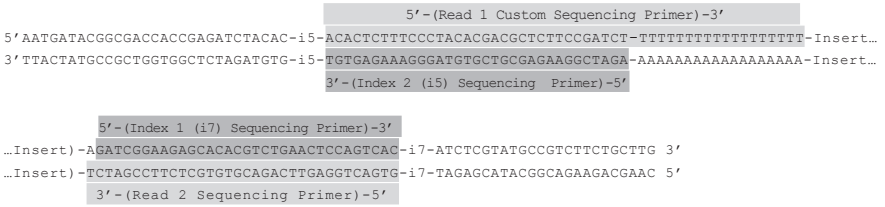
EXAMPLE: MiniSeq, iSeq, HiSeq 3000 / 4000 (PE), NovaSeq 6000 (v1.5 chemistry), NovaSeq X, and NextSeq instruments use the Reverse Complement Workflow (B) with the Multiplexing Index 2 (i5) Sequencing Primer. A minimum of eight cycles with imaging are required for i5 Index read-out, provided the correct 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 QuantSeq 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**.

QuantSeq REV V2 Libraries with dual i5 / i7 Indexing (Cat. No. 225)

i5 and i7 Indices (12 nt) are introduced during PCR (step 27).
For QuantSeq REV V2 libraries, Read 1 corresponds to the cDNA sequence.

Example for Paired-End Flow Cells - MiniSeq, iSeq, HiSeq 3000 / 4000 (PE), NovaSeq 6000 (v1.5 chemistry), NovaSeq X, and NextSeq instruments. Reverse complement workflow.



Read 1: Custom Sequencing Primer Version 5 (**CSP ●,** included):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTTTTTTTTTTTTTTT 3'

ATTENTION: As a general rule, do not use Read 1 Sequencing Primer for pure QuantSeq REV V2 lane mixes. Read 1 Sequencing Primer would result in a failed sequencing run as cluster calling would be impossible due to the poly(T) stretch.

OPTIONAL: Even though usage of **CSP ●** is highly recommended, replacing **CSP ●** with Read 1 Sequencing Primer may be attempted in specific conditions. Please contact support@lexogen.com for additional information.

ATTENTION: Do not mix **CSP ●** and Read 1 Sequencing Primer! A primer mixture would result in low clusters calls and the resulting reads would be contaminated by poly(T) stretches.

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

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

Index 2 Read (i5): i5 Index Primer (not supplied):
5' AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT 3'

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

Usage of the Custom Sequencing Primer

For QuantSeq REV V2 (Cat. No. 225) the Read 1 linker sequence is located at the 5' end of the oligo(dT) primer. Here a Custom Sequencing Primer Version 5 (**CSP ●**, included in the kit) is required for Read 1. The **CSP ●** covers the poly(T) stretch. Without the **CSP ●** cluster calling is not possible.

FORWARD THE INFORMATION BELOW for the specific sequencing instrument, along with the **CSP ●** and the lane mix to **YOUR SEQUENCING FACILITY** before starting an NGS run.

ATTENTION: Do not mix **CSP ●** and Read 1 Sequencing Primer! Do not mix **CSP ●** into HT1 or HP10! A primer mixture would result in low cluster calls and the resulting reads would be contaminated by poly(T) stretches.

MiSeq

Clustering is performed on the machine, not on the cBot. The MiSeq uses a reservoir of 600 µl with 0.5 µM sequencing primer final concentration, i.e., 3 µl of 100 µM **CSP ●** in 597 µl HT1.

NextSeq 2000

The **CSP ●** Version 5 is optimized for use on NextSeq 500 / 550 and 2000 instruments for sequencing.
Spin down the provided tube of **CSP ●** before use. Add 6 µl of 100 µM **CSP ●** to 1,994 µl of HT1 buffer (final volume 2,000 µl). Mix well and spin down. The prepared **CSP** / HT1 solution (**CSP ●** final concentration, 0.3 µM) can be then loaded into Position 7 of the NextSeq Reagent Cartridge (nextSeq500/550) or custom 1 well (NextSeq2000). Please refer to the respective NextSeq System Custom Primers Guide (Illumina) for further loading instructions.

MiniSeq and NovaSeq

Please contact support@lexogen.com if you wish to run QuantSeq REV V2 on other 2-channel instruments, including: MiniSeq, or NovaSeq.

13. Appendix H: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of QuantSeq data and is kept as general as possible for integration with your standard pipeline.
In QuantSeq REV V2 (Cat. No. 225), the Read 1 linker sequence is located at the 5' end of the oligo(dT) primer. For Read 1, a Custom Sequencing Primer (**CSP** ●, included in the kit) has to be used. With QuantSeq REV V2 it is possible to exactly pinpoint the 3' end during Read 1. The reads generated during Read 1 reflect the cDNA sequence, so they are in a strand orientation opposite to the genomic reference. Paired-end sequencing, where alignment of read pairs is required, is also possible with QuantSeq REV V2.

For more detailed information please refer to www.lexogen.com/quantseq-data-analysis and www.lexogen.com/quantseq-data-analysis-rev, respectively.

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.

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 dataset.

Trimming

Reads should be trimmed to remove adapter sequences, poly(A) / poly(T) sequences, and low quality nucleotides. Reads that are too short (i.e., <20 nt) or have generally low quality scores should be removed from the set.
In addition, for QuantSeq REV V2 libraries, as second strand synthesis is based on random priming, there is a higher proportion of mismatches over the first 12 nt of Read 2. For QuantSeq REV data we therefore recommend using an aligner that can perform soft-clipping of the read ends (e.g., STAR aligner) during alignment, or increasing the number of allowed mismatches to 14. Alternatively, trimming the first 12 nt of Read 2 can be performed prior to alignment when using a more stringent aligner (e.g., HISAT2). While trimming the read can decrease the number of reads of suitable length for alignment, the absolute number of mapping reads may increase due to the improved read quality.
Single-read sequencing using a CSP does not require this additional trimming using QuantSeq REV V2 (Cat. No. 225).

Alignment

After filtering and trimming, reads can be aligned with a short read aligner to the reference genome. We recommend the use of STAR aligner for mapping QuantSeq data (REV). The reads may not land in the last exon and span a junction, hence splice-aware aligners should be used. Bowtie2, BBMap, or BWA can also be used for mapping against a reference transcriptome.

Annotations and Read Counting

Mapping only the 3′ end of transcripts requires an annotation that covers the 3′ untranslated region (UTR) accurately. Conservative annotations might decrease the power of correct gene quantification after mapping, especially in case of QuantSeq REV V2. For some gene annotations it might be an advantage to extend the 3′ UTR annotation further downstream in order to assign the mapped read correctly.

Integrated Data Analysis on the web-based data analysis platform

With each purchased QuantSeq kit, you receive a code for free data analysis including differential expression (DE) analysis on the web-based data analysis platform (https://www.lexogen.com/data-analysis-solutions/). The activation 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 (i.e., for a 96 prep kit, 96 analysis runs can be performed). The technical parameters used for QuantSeq data analysis pipelines on the web-based data analysis platform are available online at https://www.lexogen.com/lexogen-data-analysis-solutions. Activation codes for additional pipeline runs can also be purchased from Lexogen. For further inquiries, please contact us at support@lexogen.com.

14. Appendix I: Automation

ATTENTION: Before starting any new project involving automation, we highly recommend that you reach out to our experts at support@lexogen.com.

Automating the process of library preparation has the advantage of avoiding sample tracking errors, dramatically increasing throughput, and saving hands-on time. QuantSeq is ideally suited to automation, and automated QuantSeq protocols are available for a range of liquid handling instruments, including but not limited to:

- Perkin Elmer: Sciclone® / Zephyr®
- Hamilton: Microlab STAR / STARlet / NGS STAR
- Agilent: NGS Workstation (NGS Bravo Option B)
- Beckman Coulter: Biomek FXP , Biomek i5 and Biomek i7
- Eppendorf: EpMotion® 5075
- Opentrons® OT-2

Instrument setups can vary significantly. If you are interested in automated QuantSeq scripts for these, or other liquid handling instruments not listed above, please contact us or check our online FAQs for more information.

Depending on the deck setup and script version implemented on the different liquid handling devices, QuantSeq kits will provide sufficient reagents for automated QuantSeq library preparation, or will require higher reagent volumes and script adjustments. Whatever volume is required, at Lexogen we will help you finding an appropriate solution, tailored to your needs.

Dummy reagents that mimic the QuantSeq reagent properties, designed to assist with the set-up of automated QuantSeq protocols, are available upon request.

Lexogen gladly supports implementation of Lexogen-manufactured kits, but not hardware or software issues linked to the original liquid handling instrument supplier.

15. Appendix J: Revision History

Publication No. / Revision Date	Change	Page
225UG675V0101 Dec. 15, 2023	RNA removal step 6 - incubation extended to 10 min for low input protocol.	8, 9, 15, 19, 20
	Tris and water added to user-supplied consumables list.	7
225UG675V0100 Dec. 6, 2023	Release QuantSeq 3′ mRNA-Seq REV V2 with UDIs.	

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Associated Products:

022 (Purification Module with Magnetic Beads)
025, 050, 051, 141 (SIRVs Spike-in RNA Variant Control Mixes)
028 (QuantSeq-Flex Second Strand Synthesis Module for Illumina)
070 (RS-Globin Block, *Homo sapiens*)
071 (RS-Globin Block, *Sus scrofa*)
101 - 104, 156 (Lexogen UDI 12 nt Sets A1-A4, (UDI12A_0001-0384), 1 rxn/UDI)
167 (RS-BC1 Block, *Mus musculus*)
208 (PCR Add-on and Reamplification Kit V2 for Illumina)

QuantSeq 3′ mRNA-Seq V2 Library Prep Kit REV· User Guide

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