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QUANT™ SEQ
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3' mRNA-Seq Library Prep Kit FWD with Unique Dual Indices

User Guide

Catalog Numbers:
113, 115, 129 - 131 (QuantSeq 3' mRNA-Seq Library Prep Kit FWD with UDI 12 nt Set A1, A2, A3, A4, or A1-A4)
114 (QuantSeq 3' mRNA-Seq Library Prep Kit FWD with UDI 12 nt Set B1)

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1. Overview

Lexogen's QuantSeq 3' mRNA-Seq Library Prep Kit Forward (FWD) 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) enrzchment 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 shorter read lengths: SR50 - 100.

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 (Set A: Cat. No. 113, 129 - 131, or 115; or Set B: Cat. No. 114) are included in the kit and are introduced during the PCR amplification step. Lexogen UDI 12 nt Sets are also available as separate Unique Dual Indexing Add-on Kits (Cat. No. 107 - 111) for use with other library preps such as QuantSeq Reverse (REV). For these, please refer to the Lexogen UDI 12 nt Unique Dual Indexing Add-on Kit User Guide (107UG223).

QuantSeq FWD contains the Read 1 linker sequence in the second strand synthesis primer, hence NGS reads are generated towards the poly(A) tail and directly correspond to the mRNA sequence. To pinpoint the exact 3' end, longer reads may be required. Although paired-end sequencing is possible, we do not recommend it for QuantSeq FWD. Read 2 would start with the poly(T) stretch and sequence through the homopolymer stretch, reducing the quality of Read 2.

QuantSeq FWD 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, PCR cycle optimization by qPCR assay, quality control, add-on modules, multiplexing, and sequencing guidelines are found in Appendices B - G (p.19 - 28). An automated QuantSeq bioinformatics pipeline has been integrated on the BlueBee® Genomics Platform and each purchased QuantSeq kit includes a code for free data analysis on this platform (see also Appendix H, p.29). For more details, visit our webpage at www.lexogen.com.

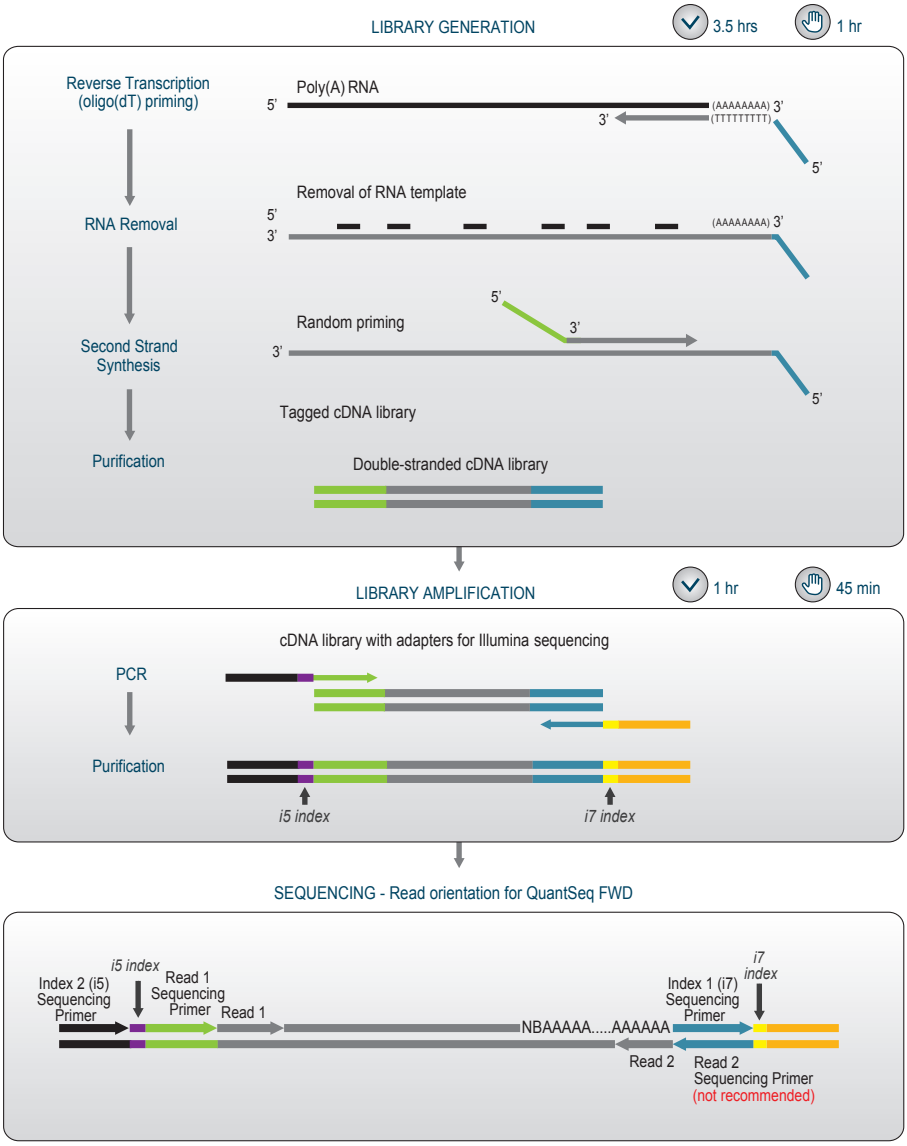

Figure 1. Schematic overview of the QuantSeq FWD with UDI library preparation workflow (Cat. No. 113 - 115, 129 - 131). Sequencing read orientation for QuantSeq FWD is depicted, where Read 1 reflects the mRNA sequence. Paired-end sequencing is not recommended for QuantSeq FWD.

2. Kit Components and Storage Conditions

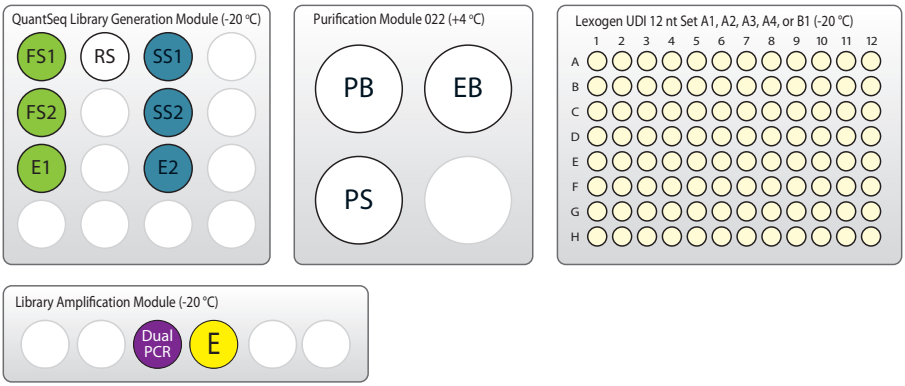

Figure 2. Location of kit components. All kits include Purification Modules. The 96 prep kits are supplied with either the UDI 12 nt Set A1, A2, A3, A4, or B1 96-well plate. The 384 prep kit contains 4 different UDI 12 nt plates (Sets A1-A4), and 4 of each module.

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 Label	Volume*		Storage
		96 preps	384 preps	
First Strand cDNA Synthesis Mix 1	FS1 ●	528 µl	4x 528 µl	-20 °C
First Strand cDNA Synthesis Mix 2	FS2 ●	1,003.2 µl	4x 1,003.2 µl	-20 °C
Enzyme Mix 1	E1 ●	52.8 µl	4x 52.8 µl	-20 °C
RNA Removal Solution	RS ○	528 µl	4x 528 µl	-20 °C
Second Strand Synthesis Mix 1	SS1 ●	1,056 µl	4x 1,056 µl	-20 °C
Second Strand Synthesis Mix 2	SS2 ●	422.4 µl	4x 422.4 µl	-20 °C
Enzyme Mix 2	E2 ●	105.6 µl	4x 105.6 µl	-20 °C
Library Amplification Module				
Dual PCR Mix	Dual PCR ●	739.2 µl	4x 739.2 µl	-20 °C
Enzyme Mix	E ●	105.6 µl	4x 105.6 µl	-20 °C
Lexogen UDI 12 nt Sets				
Lexogen UDI 12 nt Set A1, A2, A3, A4, or B1	UDI12A_0001-0096, UDI12A_0097-0192, UDI12A_0193-0288, UDI12A_0289-0384, UDI12B_0001-0096	10 µl / rxn		-20 °C
Lexogen UDI 12 nt Sets A1-A4	UDI12A_0001-0384		10 µl / rxn	-20 °C
Purification Module				
Purification Beads	PB	5,280 µl	4x 5,280 µl	+4 °C
Purification Solution	PS	10,772 µl	4x 10,772 µl	+4 °C
Elution Buffer	EB	11,616 µl	4x 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.

3. User-Supplied Consumables and Equipment

Check to 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.

Reagents / Solutions

- 80 % fresh ethanol (for washing of Purification Beads, **PB**).
- Lexogen PCR Add-on Kit for Illumina (Cat. No. 020), for qPCR assay.
- SYBR Green I (Sigma-Aldrich, Cat. No. S9430 or ThermoFisher, Cat. No. S7585), diluted to 2.5x 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

- Suitable certified ribonuclease-free 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, 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.23 for information on library quantification methods.

4. Detailed Protocol

4.1 Library Generation

Preparation

First Strand cDNA Synthesis	RNA Removal	Second Strand Synthesis	Purification
FS1 ● – thawed at RT **FS2** ● – 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 FWD is 1 ng. Input amounts ≤1 ng can also be used, please refer to Appendix B, p.19 for protocol modifications.

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

NOTE: If longer inserts or larger RNA input volumes (up to 13 µl) are required, the QuantSeq-Flex First Strand Synthesis Module V2 (Cat. No. 166) can be used. In this case, skip steps 1 to 4 in the protocol below, and refer instead to steps 1 to 4 of the QuantSeq-Flex User Guide (166UG337), then, continue from step 5 below.
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 5 µl, with 5 µl First Strand cDNA Synthesis Mix 1 (**FS1** ●) in a PCR plate. If necessary, 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. **REMARK:** Skip this step for low input / low quality / FFPE RNA. Do not add **FS1** ● to the RNA. Place the RNA samples briefly at room temperature and proceed to step 3.

2 Denature the RNA / **FS1** 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. **REMARK:** Skip this step for low input / low quality / FFPE RNA.

3 Prepare a mastermix containing 9.5 µl First Strand cDNA Synthesis Mix 2 (**FS2** ●) 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. **REMARK:** If step 2 is skipped, prepare a mastermix containing 5 µl **FS1** ●, 9.5 µl **FS2** ●, and 0.5 µl **E1** ● per sample. Mix well, spin down, and pre-warm for 2 - 3 minutes at 42 °C. **ATTENTION:** Do not cool mastermixes on ice.

4 Quickly spin down the denatured RNA / **FS1** 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 **FS2** / **E1** mastermix to each reaction, mix well, and seal the plate. Spin down briefly and incubate the reactions for 15 minutes at 42 °C. **REMARK:** If step 2 is skipped, add 15 µl of the pre-warmed **FS1** / **FS2** / **E1** mastermix to each 5 µl RNA sample, mix well, and seal the plate. Spin down briefly and incubate the reactions for 15 minutes at 42 °C. **OPTIONAL:** For low input / low quality / FFPE RNA, extend the incubation time to 1 hour at 42 °C. **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).

Start here if continuing on from step 4 of the QuantSeq-Flex First Strand Synthesis Protocol (166UG323).

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: At step 7 the UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1, Cat. No. 081) may be used to include Unique Molecular Identifiers (UMIs) in QuantSeq FWD libraries. The UMI Second Strand Synthesis Mix (**USS** ●) replaces the Second Strand Synthesis Mix 1 (**SS1** ●) from the standard QuantSeq FWD Kits (Cat. No. 113 - 115, 129 - 131). For further information please consult the UMI Module for QuantSeq User Guide (081UG366).

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. **REMARK:** 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. Make sure that accumulated beads are not disturbed.
15	Add 40 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.
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. **REMARK:** 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	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.
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 with 12 nt Unique Dual Indices (UDIs)

This section describes unique dual indexing PCR for multiplexing up to 384 libraries using the Lexogen 12 nt UDIs included in these kits.

Preparation

PCR			Purification (Cat. No. 022)*	
Dual PCR ●		- thawed at RT	**PB**	– stored at +4 °C
E ●		- keep on ice or at -20 °C	**PS**	– stored at +4 °C
Lexogen UDI 12 nt Sets (A1 - A4, or B1)		- thawed at RT	**80 % EtOH**	– provided by user **prepare fresh!**
		spin down before opening!	**EB**	– stored at +4 °C
Thermocycler	98 °C, 30 sec		Equilibrate all reagents to room temperature for 30 minutes prior to use.	
	98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec	11- 25x Endpoint cycle number as determined by qPCR (Cat. No. 020), see Appendix D, p.21.		
	72 °C, 1 min			
	10 °C, ∞			

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 Kit for Illumina (Cat. No. 020) is required. For qPCR assay details, see Appendix D, p.21.
- 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 Dual PCR Mix (**Dual PCR** ●) and 1 µl Enzyme Mix (**E** ●) per reaction.
26	Add 8 µl of the **Dual PCR / E** 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 11 - 25 cycles of PCR (as determined by qPCR, see Appendix D, p.21) with the following program: Initial denaturation at 98 °C for 30 seconds, 11 - 25 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, hold at 10 °C. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The 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. **REMARK:** 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	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.
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) as this will negatively influence the elution and the resulting library yield.

39 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.

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.23), pooling (for multiplexing, Appendix F, p.25), 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) / Low Quality / FFPE
First Strand cDNA Synthesis	
☐ Mix 5 µl RNA and 5 µl **FS1** ●.	☐ Skip!
☐ Incubate for 3 min at 85 °C, then cool to 42 °C. **Keep samples on thermocycler at 42 °C!**	☐ Skip!
☐ Prepare a mastermix with 9.5 µl **FS2** ● and 0.5 µl **E1** ● per reaction, mix well, and pre-warm for 2 - 3 min at 42 °C.	☐ Prepare a mastermix with 5 µl **FS1** ●, 9.5 µl **FS2** ●, and 0.5 µl **E1** ● per reaction. Mix well and pre-warm at 42 °C. **Place RNA samples at RT for 2 - 3 min.**
☐ Add 10 µl **FS2 / E1** mix per reaction, mix well, and spin down. **Keep samples on thermocycler at 42 °C when adding mastermix!**	☐ Add 15 µl **FS1 / FS2 / E1** mix to each 5 µl RNA sample, mix well, and spin down. **Transfer samples to thermocycler at 42 °C!**
☐ Incubate for 15 min at 42 °C. **Proceed immediately to RNA Removal!**	☐ Incubate for 15 min (**or** 1 hr) 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.	
☐ Add 40 µl **EB**, remove from magnet, 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!	
☐ 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 before opening tubes or plates!

1.2 hrs (+qPCR) Library Amplification

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

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

- ☐ Add 2 µl of **EB** to the 17 µl of eluted cDNA.
- Prepare a 2.5x stock of SYBR Green I nucleic acid stain (i.e., 1:4,000 dilution in DMSO; use Sigma-Aldrich, Cat. No. S9430 or ThermoFisher, Cat. No. S7585).
- ☐ Combine 1.7 µl of cDNA with: 7 µl **PCR** ●, 5 µl Primer **7000**, 1 µl **E** ● (from PCR Add-on Kit), 1.2 µl of 2.5x SYBR Green I nucleic acid stain, and 14.1 µl of **EB**, per reaction. Mix well.
- ☐ PCR: 98 °C, 30 sec

  98 °C, 10 sec
  65 °C, 20 sec
  72 °C, 30 sec
  } **35x** (see p.21)

  72 °C, 1 min

  10 °C, ∞. Calculate the optimal cycle number for Endpoint PCR (see Appendix D, p.21).

Endpoint PCR

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

  98 °C, 10 sec
  65 °C, 20 sec
  72 °C, 30 sec
  } **11 - 25x** (see p.21)

  72 °C, 1 min

  10 °C, ∞. ☞ Safe stopping point.

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

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.
- ☐ 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 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 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 FWD. 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 (500 pg - 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.
- 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 FWD 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 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** (see Appendix D, p.21). 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**
0.5 ng*	21 - 25
10 ng*	17 - 20
100 ng	14 - 17
≥500 ng	11 - 14

* Using low input protocol modifications with 1 hour incubation at 42 °C at step 4 (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 (See Appendix D, p.21).

7. Appendix B: Low Input RNA

All protocol modifications that apply when using low input RNA (≤10 ng), low quality or degraded RNA, or RNA isolated from formalin-fixed, paraffin-embedded (FFPE) sample, 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 ≤1 ng of 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 **FS1** to RNA samples. Do not place samples back on ice after adding **FS1** !	**Skip denaturation step!** Place RNA samples briefly at room temperature while the mastermix is prepared (**2-3 min**).	
Step 2	Incubate for 3 minutes at 85 °C, then cool to 42 °C. Hold samples at 42 °C on the thermocycler.		
Step 3	Prepare **FS2 / E1** mastermix – pre-warm for 2 - 3 minutes at 42 °C.	Prepare **FS1 / FS2 / E1** mastermix – pre-warm for 2 - 3 minutes at 42 °C.	
Step 4	Add pre-warmed mastermix to **RNA / FS1** samples on the thermocycler at 42 °C. Incubate for 15 minutes at 42 °C.	Add pre-warmed mastermix to RNA samples at room temperature and transfer to a thermocycler preheated to 42 °C. Incubate for 15 minutes at 42 °C, or increase incubation time to 1 hour.	
Step 6	Incubate for 10 minutes at 95 °C.	Incubate for 10 minutes at 95 °C.	Incubate for 5 minutes at 95 °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 (see Appendix D, p.21). 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 are 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 (see also Appendix B, p.19). Specifically:

- Skipping steps 1 and 2 and preparing a mastermix of **FS1 / FS2 / E1**.
- Reducing the volume of **PS** in step 16 to 48 µl.
- Reducing the volume of **PB** in step 29 to 27 µl for single indexing PCR, and 31.5 µl in step 30 for dual indexing PCR, respectively.

Further optional adjustments for low input FFPE RNA samples may also be included, such as:

- Extending the reverse transcription time in step 4 to 1 hour (≤10 ng).
- Reducing the RNA removal time in step 6 to 5 minutes at 95 °C (≤1 ng).

As the RNA amount is often a limiting factor with FFPE samples, QuantSeq was tested with 500 pg - 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 PCR Add-on Kit for Illumina (Cat. No. 020), Appendix D, p.21) 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.23). 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, re-purification 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.
- 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: cDNA Quantification by qPCR and Library Reamplification

Adjusting PCR Cycle Numbers for Sample Type

The mRNA content and quality of total RNA affects the number of PCR cycles needed for the final library amplification step. Variable input types and amounts require optimization of PCR cycle numbers (see Appendix A, p.18). **We strongly recommend taking advantage of the qPCR assay to optimize the number of cycles required for the endpoint PCR.** This will prevent both under and overcycling, the latter of which may bias your sequencing results (see also Appendix E, p.23).

The mRNA content of RNA samples can vary between species and tissue / cell types. Variable RNA quality, particularly for FFPE RNA samples, may also affect differences in mRNA content between samples.

The PCR Add-on Kit for Illumina (Cat. No. 020) is required for the following qPCR assay protocol. This assay can be used to determine cycle numbers for subsequent dual or single indexing PCRs.

qPCR to Determine the Optimal Cycle Number for Endpoint PCR

The PCR Add-on Kit provides additional PCR Mix (**PCR** ●), Enzyme Mix (**E** ●), and the P7 Primer (**7000** ●) required for the qPCR assay. In addition, SYBR Green I nucleic acid dye (Sigma Aldrich, S9430 or ThermoFisher, Cat. No. S7585) is also needed and must be supplied by the user.

ATTENTION:

- Do not use the 12 nt UDIs with the PCR Mix (**PCR** ●) from the PCR Add-on Kit for Illumina (Cat. No. 020)!
- The use of SYBR Green I-containing qPCR mastermixes from other vendors is not recommended.

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

1	Dilute the double-stranded library from step 24 to 19 µl by adding 2 µl Elution Buffer (**EB**) or molecular biology-grade water.
2	Prepare a 1:4,000 dilution of SYBR Green I dye in DMSO, for a 2.5x working stock concentration. **ATTENTION:** The final concentration in the reaction should be 0.1x. Higher concentrations of SYBR Green I will inhibit amplification.
3	For each reaction combine: 1.7 µl of the diluted cDNA library, 7 µl of PCR Mix (**PCR** ●), 5 µl of P7 Primer (**7000** ●), 1 µl of Enzyme Mix (**E** ●), and 1.2 µl of 2.5x SYBR Green I nucleic acid dye. Make the total reaction volume up to 30 µl by adding 14.1 µl of Elution Buffer (**EB**) or molecular biology-grade water. **ATTENTION:** Include a no template control!

4 Perform 35 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, 35 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, hold at 10 °C. **REMARK:** There is no need to purify or analyze the overcycled PCR reaction on a Bioanalyzer.

5 Using the amplification curves in linear scale, determine the value at which the fluorescence reaches the plateau. Calculate 50 % of this maximum fluorescence value and determine at which cycle this value is reached. As the endpoint PCR will contain 10x more cDNA compared to the qPCR, subtract three from this cycle number. This is then the final cycle number you should use for the endpoint PCR with the remaining 17 µl of the template (see Fig. 3).

Endpoint PCR Cycle Calculation

When using 1.7 µl of cDNA for a qPCR, if the cycle number corresponding to 50 % of the maximum fluorescence is 15 cycles, the remaining 17 µl of the template should therefore be amplified with 12 cycles (15 - 3 cycles =12 cycles, Fig. 3).

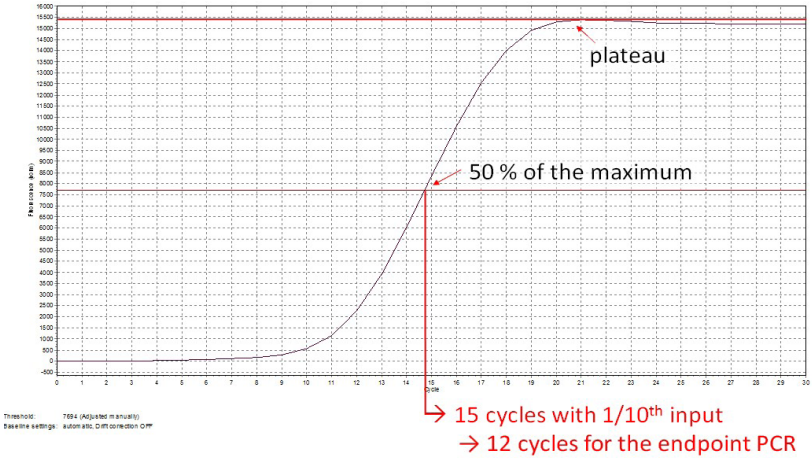

Figure 3. Calculation of the number of cycles for the endpoint PCR.

NOTE: Once the number of cycles for the endpoint PCR is established for one type of sample (same input amount, tissue / cell type, and RNA quality), there is no need for further qPCRs. The entire cDNA can be inserted straight into the endpoint PCRs.

Reamplification of Uniquely Dual-Indexed Libraries

If your library yields are extremely low and insufficient for pooling, reamplification can be performed. Dual-indexed libraries must be reamplified using the Reamplification Add-on Kit for Illumina (080.96). This kit is available only upon request. Please contact Lexogen at support@lexogen.com for more information.

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 (e.g., FFPE samples typically produce shorter libraries than high quality Universal Human Reference RNA (UHRR), see Figures 4 and 5). The majority of inserts are greater than 75 bp in size, corresponding to final library fragment sizes ≥200 bp.

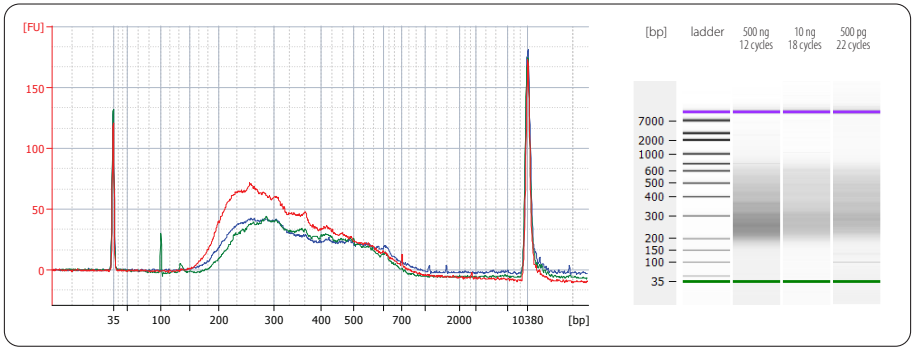

Figure 4. Bioanalyzer traces of QuantSeq FWD libraries prepared from different input amounts of total RNA input (UHRR). Libraries were prepared with the standard protocol, using 500 ng (red trace, 12 PCR cycles). Libraries with 10 ng (blue trace, 18 PCR cycles) and 500 pg (green trace, 22 PCR cycles) of UHRR input were prepared using low input protocol modifications (see Appendix B, p.19; reverse transcription for 1 hour at 42 °C for both, 5 minute incubation at 95 °C for 500 pg). Endpoint PCR was performed using the non-indexed P7 Primer 7000 (from the PCR Add-on Kit, Cat. No. 020).

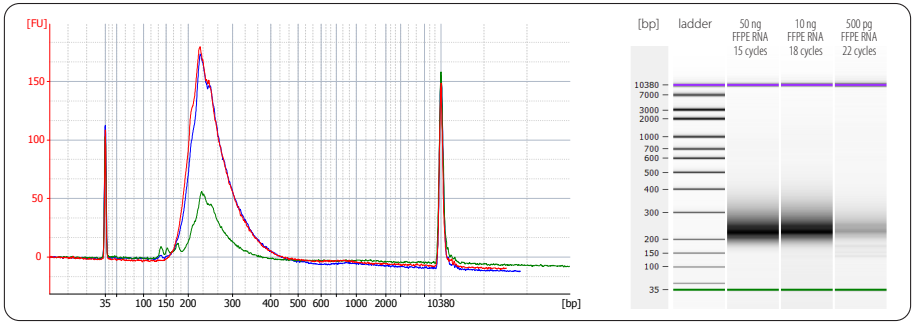

Figure 5. Bioanalyzer traces of QuantSeq FWD libraries synthesized from 50 ng (red trace), 10 ng (dark blue trace), and 500 pg (green trace), using mouse (Mm) brain FFPE RNA (RIN 1.8, DV_{200} 51 %) as input. All libraries were prepared with the recommendations for FFPE RNA input (Appendix C, p.20). 500 pg FFPE RNA libraries already contain some artifacts below 175 bp which should be removed before sequencing, e.g., by repurifying the lane mix (see Appendix F, p.26).

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 Appendix D, p.21.

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)	**Sets A1, A2, A3, and/or A4** (UDI12A_0001-0384) QuantSeq 3′ mRNA-Seq: Cat. No. 113, 129, 130, 131, or 115
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) QuantSeq 3′ mRNA-Seq: Cat. No. 114

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 FWD 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.14).

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 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 FWD 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 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 FWD libraries:

```
                                    5’-(Read 1 Sequencing Primer)-3’
5’AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert…
3’TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert…
                                    3’-(Index 2 (i5) Sequencing  Primer)-5’

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

Sequencing Primers

Standard Illumina sequencing primers are used for all dual-indexed libraries.
The Multiplexing Read 1 Sequencing Primer is always used for Read 1 sequencing, and the Index 1 (i7) Sequencing Primer is always used for Index 1 Read (i7) sequencing. The Index 2 Read (i5) is initiated using different sequencing primers specific to the instrument and flow cell type. For more details, please refer to the User Guide for Lexogen 12 nt Unique Dual Indexing Add-on Kits (107UG223) or contact support@lexogen.com.

Read 1 for QuantSeq FWD, 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’

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

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.
QuantSeq FWD (Cat. No. 015, 113 - 115, 129 - 131) contains the Read 1 linker sequence in the 5' part of the second strand synthesis primer, hence NGS reads are generated towards the poly(A) tail. To pinpoint the exact 3' end, longer read lengths may be required. Read 1 directly reflects the mRNA sequence. For more detailed information, please refer to www.lexogen.com/quant-seq-data-analysis.

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 both demultiplexing and 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

The 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 FWD libraries, as second strand synthesis is based on random priming, there is a higher proportion of mismatches over the first 12 nt of the reads. For QuantSeq FWD 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 1 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.

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 (FWD). 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. 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 Pipeline at BlueBee®

Each purchased QuantSeq kit includes a code for free data analysis including differential expression (DE) analysis using the BlueBee® Genomics Platform (for fastq(.gz) file sizes up to 1.5 GB). The activation code can be found on a card within the Library Generation Module (stored at -20 °C). 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). Activation codes for additional pipeline runs can also be purchased from Lexogen (Cat. No. 090, 091, 093, and 094). The FWD-specific pipeline is automatically encoded in the allocated activation code supplied with the respective QuantSeq Kit, ensuring that the correct pipeline parameters are used for the analysis.

To register yourself as a first time user of the BlueBee® Analysis Platform, go to: https://lexogen.bluebee.com/quantseq. For further inquiries, please contact us at support@lexogen.com.

Details of the technical parameters used for QuantSeq data analysis pipelines on the BlueBee® platform are provided in the QuantSeq 3′ mRNA-Seq Integrated Data Analysis Pipeline on BlueBee® Platform User Guide, available online at https://www.lexogen.com/lexogen-data-analysis-solutions-on-bluebee-platform/.

14. Appendix I: Automation

Automating the process of library preparation has the advantage of avoiding sample tracking errors, dramatically increasing throughput, and saving hands-on time. QuantSeq is ideally suited to automation, and autoQuantSeq 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 FX^P and Biomek i7
- Eppendorf: EpMotion® 5075

Instrument setups can vary greatly, so if you are interested in autoQuantSeq scripts for these, or other liquid handling instruments not listed, please contact us at support@lexogen.com or check our online FAQs for more information.

ATTENTION: For QuantSeq 3′ mRNA-Seq Library Prep Kit FWD with UDIs, the following volume changes need to be considered for automation:

- Instead of 5 µl of i7 index primer, 10 µl UDI primer mix is added.
- During the post-PCR purification, 35 µl of **PB** are added for standard RNA inputs (>10 ng), and 31.5 µl of **PB** for low input (≤10 ng) / low quality / FFPE RNA.

The QuantSeq with UDI 12 nt kits provide sufficient reagents for autoQuantSeq library preparation, hence there is no separate kit for autoQuantSeq Library Prep.

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

15. Appendix J: Revision History

Publication No. / Revision Date	Change	Page
113UG227V0110 Aug. 16, 2021	Updated legal information.	2
	Removed General Guidelines.	8
	Updated QuantSeq-Flex information.	9
	Updated recommended input range to 1 ng - 500 ng.	9 - 10, 18
	Updated references to Globin and BC1 Block solutions, and UMI Module for QuantSeq (removal of dedicated Appendices).	10 - 17
	Streamlined information in Appendices.	18 - 37
	Updated linker-linker information.	20, 24, 30
	Updated dumultiplexing instructions.	35
113UG227V0100 Dec. 27, 2019	Initial Release	

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Associated Products:

020 (PCR Add-on Kit for Illumina)
022 (Purification Module with Magnetic Beads)
025, 050, 051, 141 (SIRVs Spike-in RNA Variant Control Mixes)
028 (QuantSeq-Flex Second Strand Synthesis Module for Illumina)
070 (RS-Globin Block, *Homo sapiens*)
071 (RS-Globin Block, *Sus scrofa*)
080 (Reamplification Add-on Kit for Illumina)
081 (UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1))
090 - 094 (QuantSeq Data Analysis on BlueBee® Genomics Platform)
107 - 111, 120 (Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits, Set A1, A2, A3, A4, A1 - A4, or B1)
166 (QuantSeq-Flex First Strand Synthesis Module V2 for Illumina)
167 (RS-BC1 Block, *Mus musculus*)

QuantSeq 3′ mRNA-Seq Library Prep Kit FWD with UDIs · User Guide

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