

The background of the entire page is decorated with a network of translucent blue spheres of various sizes, connected by thin, light blue lines. The spheres have a glossy, 3D appearance with highlights and shadows. The lines form a web-like structure across the page.

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3' mRNA-Seq Library Prep Kit User Guide

Catalog Numbers:

015 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (FWD))

016 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (REV) with Custom Sequencing Primer)

015UG009V0260



Important recommendations for QuantSeq 3' mRNA Library Prep

Critical Steps for QuantSeq 3' mRNA-Seq Library Prep Kits!

First Strand cDNA Synthesis (p.8-9):

1. At step **3** pre-warm the FS2 / E1 mastermix for 2 - 3 minutes at 42 °C. Do not cool the mastermix on ice!
2. Have the RNA / FS1 samples at 42 °C when adding the pre-warmed FS2 / E1 mastermix (step **4**) and mix properly when adding. Any drop in temperature at this point can cause mishybridization. Seal the plate or tubes and begin the 42 °C incubation.
3. Spin down the samples at room temperature before and after adding the FS2 / E1 mastermix.

If step **2** is skipped for low input or degraded samples (≤10 ng, or FFPE samples):

1. Prepare your RNA samples in 5 µl volumes.
2. Prepare a mastermix containing 5 µl FS1, 9.5 µl FS2, and 0.5 µl E1, mix well, spin down, and pre-warm at 42 °C on a thermocycler for 2 - 3 minutes.
3. Bring your RNA samples to room temperature while the mastermix is pre-warming.
4. Spin down the pre-warmed FS1 / FS2 / E1 mastermix and add 15 µl to each RNA sample. Mix, seal the plate or strip-tubes, spin down briefly at room temperature, and then commence the 42 °C incubation for 15 minutes (or 1 hour for low input RNA (≤10 ng)).
5. Proceed immediately to RNA Removal.

Protocol modifications for low input (≤10 ng), FFPE, or low quality / degraded RNA versus standard RNA input:

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 minutes).	
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.	

Turn me over

Protocol Step	Standard Input (>10 ng)	Low Input (≤10 ng)* FFPE / Degraded RNA	Low Input (≤1 ng)*
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	<p>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.20). The qPCR assay should be performed also when RNA samples are of:</p> <ul style="list-style-type: none">• 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 30 µl of Purification Beads (PB) for single-indexed libraries, or 35 µl for dual-indexed libraries.	Reduce volume of Purification Beads (PB) to 27 µl for single-indexed libraries, or 31.5 µl for dual-indexed libraries.	

*Only for QuantSeq FWD. Minimum input for QuantSeq REV is 10 ng.

Sequencing Recommendations for QuantSeq Libraries

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).

For further details please contact support@lexogen.com.

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When describing a procedure for publication using this product, please refer to it as Lexogen's QuantSeq™ 3' mRNA-Seq Kit.

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Table of Contents

1. Overview.	4
2. Kit Components and Storage Conditions	6
3. User-Supplied Consumables and Equipment	7
4. Detailed Protocol	8
4.1 Library Generation	8
4.2. Library Amplification - Single Indexing (i7 only).	12
5. Short Procedure.	15
6. Appendix A: RNA Input and PCR Cycles.	17
7. Appendix B: Low Input RNA	18
8. Appendix C: Low Quality RNA - FFPE	19
9. Appendix D: qPCR and Library Reamplification	20
10. Appendix E: Library Quality Control.	23
11. Appendix F: Multiplexing	25
12. Appendix G: Sequencing*	28
13. Appendix H: Data Analysis	32
14. Appendix I: Automation	34
15. Appendix J: Revision History	35

1. Overview

Lexogen's QuantSeq 3' mRNA-Seq Kits provide library preparation protocols that 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 the transcripts.

QuantSeq is available with two read directions, forward (FWD) and reverse (REV). QuantSeq Forward (FWD, Cat. No. 015) 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. For QuantSeq Reverse (REV, Cat. No. 016) 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.

Both QuantSeq FWD and REV maintain strand-specificity and 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. Multiplexing of libraries can be carried out using up to 96 i7 indices (included). Additional i5 6 nt Dual Indexing Add-on Kits (Cat. No. 047) are also available for preparing and multiplexing up to 9,216 uniquely-barcoded libraries. QuantSeq Library Prep Kits (FWD and REV) are also compatible with UDI 12 nt Unique Dual Indexing Add-on Kits (Cat. No. 107 - 111, 120). For convenience, QuantSeq 3' FWD Library Prep Kits with Unique Dual Indices are also available (Cat. No. 113 - 115, 129 - 131).

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.17. Library generation is initiated by oligo(dT) priming (Fig. 1). The primer already contains 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 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 (FWD and REV) or PE50 - 100 (REV). Second strand synthesis is followed by a magnetic bead-based purification step. The library is then amplified, introducing the sequences required for cluster generation. i7 indices for multiplexing are included in all QuantSeq kits and are introduced during the PCR amplification step.

Appendices B - I contain additional information on protocol modifications, PCR cycle optimization by qPCR assay, quality control, add-on modules, multiplexing, and sequencing guidelines. An automated QuantSeq bioinformatics pipeline has been integrated on the BlueBee® Genomics Analysis Platform and each purchased QuantSeq kit includes a code for free data analysis on this platform (see also Appendix H, p.32). For more details visit our webpage at www.lexogen.com.

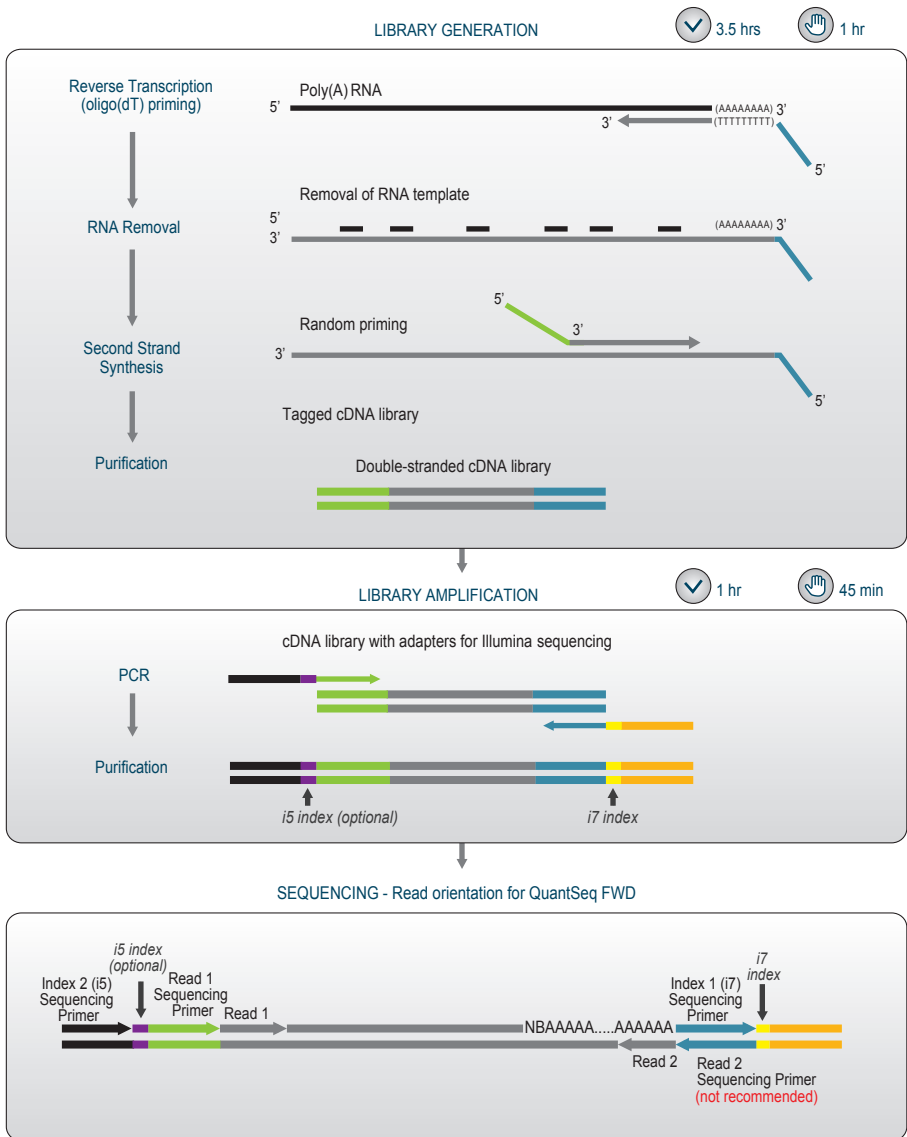


Figure 1. Schematic overview of the QuantSeq FWD library preparation workflow (Cat. No. 015). For QuantSeq REV (Cat. No. 016) the position of adapters for Read 1 (green) and Read 2 (blue) are switched. Sequencing read orientation for QuantSeq FWD and QuantSeq REV is depicted as well. For QuantSeq FWD, Read 1 reflects the mRNA sequence. Paired-end sequencing is not recommended for QuantSeq FWD. QuantSeq REV is suitable for paired-end sequencing, and Read 1 reflects the cDNA sequence. A Custom Sequencing Primer (CSP Version 5, included in the kit) is required for Read 1.

2. Kit Components and Storage Conditions

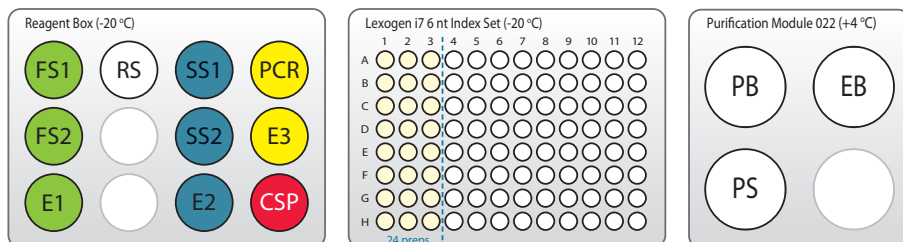


Figure 2. Location of kit components. For the 24 prep kit the Lexogen i7 6 nt Index Set is only filled with indices 7001-7024 (up to the blue dotted line). CSP (red lid color) is only required and included for QuantSeq REV (Cat. No. 016.24, 016.96). All kits include Purification Modules.

Kit Component	Tube Label	Volume*		Storage
		24 preps	96 preps	
First Strand cDNA Synthesis Mix 1	FS1 ●	132 µl	528 µl	-20 °C
First Strand cDNA Synthesis Mix 2	FS2 ●	250.8 µl	1,003.2 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	1056 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
PCR Mix	PCR ●	184.8 µl	739.2 µl	-20 °C
Enzyme Mix 3	E3 ●	26.4 µl	105.6 µl	-20 °C
Lexogen i7 6 nt Index Set (96-well plate)		5 µl / reaction		-20 °C
Custom Sequencing Primer Version 5 (100 µM)**	CSP ●	25 µl	50 µl	-20 °C
Purification Module (Cat. No. 022) included in the kit				
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

** only required for QuantSeq REV (Cat. No. 016)

*including ≥10 % surplus

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.

ATTENTION: The Custom Sequencing Primer Version 5 (**CSP ●**) is only required for QuantSeq REV (Cat. No. 016) libraries. **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.28. **Forward this information to your sequencing facility before starting a sequencing run.**

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.19, 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 be used, but please refer to Appendix B, p.18 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.18, and Appendix C, p.19).

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 First Strand cDNA Synthesis Mix 1 (**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 Kit (Cat. No. 015). 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. **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. 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.

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.18 and Appendix C, p.19).

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 - Single Indexing (i7 only)

This section describes single indexing PCR (i7 indices only) for multiplexing and unique indexing of up to 96 libraries using the Lexogen i7 6 nt Index Set included in the kit. Lexogen also offers i5 Dual Indexing Add-on Kits (Cat. No. 047), which enable dual indexing for up to 9,216 different i5/i7 index combinations, or preparation of 96 libraries with unique dual indexing. For details, please refer to the i5 Dual Indexing Add-on Kits User Guide (047UG109).

NOTE: QuantSeq Library Prep Kits (FWD and REV) are compatible with Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits. For details, please refer to the Lexogen 12 nt Unique Dual Indexing Add-on Kits User Guide (107UG223).

Preparation

PCR	Purification
PCR ● E3 ● i7 6 nt Index Set – thawed at RT – keep on ice or at -20 °C – thawed at RT; spin down before opening!	PB – stored at +4 °C PS – stored at +4 °C 80 % EtOH – provided by user; prepare fresh! EB – stored at +4 °C
Thermocycler 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞ } 12 - 26x Appendix D, p.20	Equilibrate all reagents to room temperature for 30 minutes prior to use.

PCR


The library is amplified to add the complete adapter sequences required for cluster generation and unique indices for multiplexing, and to generate sufficient material for quality control and sequencing.

ATTENTION: Important notes for Library Amplification.

- **Perform a qPCR assay to determine the optimal PCR cycle number for endpoint PCR.**
The number of PCR cycles for library amplification must be adjusted according to RNA input amount, quality, and sample type. The PCR Add-on Kit for Illumina (Cat. No. 020) is required. For qPCR assay details see Appendix D, p.20.
- Avoid cross contamination when using the Lexogen i7 6 nt Index Set. 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 indices only. Reseal opened wells after use to prevent cross contamination.
- Each well of the Lexogen i7 6 nt Index Set is intended for single use only.

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

OPTIONAL: For unique dual indexing with the Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits (Cat. No. 107 - 111, 120), **do not** use **PCR** ●! Please refer to the respective User Guide (107UG223).

- 25 Prepare a mastermix containing 7 µl of PCR Mix (**PCR** ●) and 1 µl Enzyme Mix 3 (**E3** ●) per reaction, mix well by pipetting, spin down.
- 26 Add 8 µl of the **PCR / E3** mastermix to 17 µl of the eluted library.
- 27 Add 5 µl of the respective i7 index (**7001-7096**, in 96-well plate). Mix well by pipetting. Seal the PCR plate and quickly spin down. **ATTENTION:** Reseal opened wells of the Lexogen i7 6 nt Index Set after use to prevent cross contamination.
- 28 Conduct 11 - 25 cycles of PCR (as determined by qPCR, see Appendix D, p.20) with: 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.

- For **QuantSeq** libraries (**standard input >10 ng**), add 30 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 27 µl **PB**. Mix well, and incubate for 5 minutes at room temperature.
- 29
 - 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 μ l of 80 % EtOH, and incubate the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.
-
- 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 Add 20 μ l of Elution Buffer (**EB**) per well, remove the plate from the magnet, and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
-
- 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 μ l 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	
<input type="checkbox"/> Mix 5 μ l RNA and 5 μ l FS1 ●.	<input type="checkbox"/> Skip!
<input type="checkbox"/> Incubate for 3 min at 85 °C, then cool to 42 °C. Keep samples on thermocycler at 42 °C!	<input type="checkbox"/> Skip!
<input type="checkbox"/> Prepare a mastermix with 9.5 μ l FS2 ● and 0.5 μ l E1 ● per reaction, mix well and pre-warm for 2 - 3 min at 42 °C.	<input type="checkbox"/> Prepare a mastermix with 5 μ l FS1 ●, 9.5 μ l FS2 ●, and 0.5 μ l E1 ● per reaction, mix well and pre-warm for 2 - 3 min at 42 °C. Place RNA samples at RT for 2 - 3 min.
<input type="checkbox"/> Add 10 μ l FS2 / E1 mix per reaction, mix well, and spin down. Keep samples on thermocycler at 42 °C when adding mastermix!	<input type="checkbox"/> Add 15 μ l FS1 / FS2 / E1 mix to each 5 μ l RNA sample, mix well and spin down. Transfer samples to thermocycler at 42 °C!
<input type="checkbox"/> Incubate for 15 min at 42 °C. Proceed immediately to RNA Removal!	<input type="checkbox"/> Incubate for 15 min (or 1 hr) at 42 °C. Proceed immediately to RNA Removal!
RNA Removal	
<input type="checkbox"/> Add 5 μ l RS ○, mix well.	
<input type="checkbox"/> Incubate 10 min at 95 °C, cool to 25 °C.	
Second Strand Synthesis	
<input type="checkbox"/> Add 10 μ l SS1 ●, mix well.	
<input type="checkbox"/> Incubate 1 min at 98 °C, slowly ramp down to 25 °C (0.5 °C / sec).	
<input type="checkbox"/> Incubate 30 min at 25 °C.	
<input type="checkbox"/> Prepare a mastermix with 4 μ l SS2 ● and 1 μ l E2 ● per reaction, mix well.	
<input type="checkbox"/> Add 5 μ l SS2 / E2 mix per reaction, mix well.	
<input type="checkbox"/> Incubate 15 min at 25 °C. 🛑 Safe stopping point.	
Purification	
<input type="checkbox"/> Add 16 μ l PB per reaction, mix well, incubate 5 min at RT.	
<input type="checkbox"/> Place on magnet for 2 - 5 min, discard supernatant.	
<input type="checkbox"/> Add 40 μ l EB , remove from magnet, mix well, incubate 2 min at RT.	
<input type="checkbox"/> Add 56 μ l PS , mix well, incubate 5 min at RT.	<input type="checkbox"/> For low input / low quality / FFPE: Add 48 μ l PS , mix well, incubate 5 min at RT.
<input type="checkbox"/> Place on magnet for 2 - 5 min, discard supernatant.	
<input type="checkbox"/> Rinse beads twice with 120 μ l 80 % EtOH, 30 sec.	
<input type="checkbox"/> Air dry beads for 5 - 10 min. ATTENTION: Do not let the beads dry too long!	
<input type="checkbox"/> Add 20 μ l EB , remove from magnet, mix well, incubate 2 min at RT.	
<input type="checkbox"/> Place on magnet for 2 - 5 min, transfer 17 μ l 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)]	
<input type="checkbox"/> 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).	
<input type="checkbox"/> 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.	
<input type="checkbox"/> PCR: 98 °C, 30 sec. <div style="display: flex; align-items: center; margin-left: 40px;"> <div style="display: flex; flex-direction: column; align-items: center;"> <div>98 °C, 10 sec</div> <div>65 °C, 20 sec</div> <div>72 °C, 30 sec</div> </div> <div style="font-size: 3em; margin: 0 10px;">}</div> <div style="text-align: left;"> 35x p.20 </div> </div> <div style="margin-left: 40px;">72 °C, 1 min</div> <div style="margin-left: 40px;">10 °C, ∞. Calculate the optimal cycle number for Endpoint PCR (see Appendix D, p.20).</div>	
Endpoint PCR	
<input type="checkbox"/> Prepare a mastermix with 7 µl PCR ● and 1 µl E3 ● per reaction, mix well.	
<input type="checkbox"/> Add 8 µl PCR / E3 mastermix to 17 µl of the eluted library.	
<input type="checkbox"/> Add 5 µl i7 Primer (7001-7096 , from the 96-well plate) to each reaction, mix well. ATTENTION: Reseal opened index wells after use!	
<input type="checkbox"/> PCR: 98 °C, 30 sec <div style="display: flex; align-items: center; margin-left: 40px;"> <div style="display: flex; flex-direction: column; align-items: center;"> <div>98 °C, 10 sec</div> <div>65 °C, 20 sec</div> <div>72 °C, 30 sec</div> </div> <div style="font-size: 3em; margin: 0 10px;">}</div> <div style="text-align: left;"> 11 - 25x p.20 </div> </div> <div style="margin-left: 40px;">72 °C, 1 min</div> <div style="margin-left: 40px;">10 °C, ∞. 🛑 Safe stopping point.</div> <div style="margin-left: 100px; margin-top: 10px;"> ATTENTION: Increased cycle numbers may be required for low input / low quality / FFPE RNA (see Appendix B, p.18 and Appendix C, p.19) </div>	
Purification	
<input type="checkbox"/> Add 30 µl PB per reaction, mix well, incubate 5 min at RT.	<input type="checkbox"/> For low input / low quality / FFPE: Add 27 µl PB per reaction, mix well, incubate 5 min at RT.
<input type="checkbox"/> Place on magnet for 2 - 5 min, discard supernatant.	
<input type="checkbox"/> Add 30 µl EB , remove from magnet, mix well, incubate 2 min at RT.	
<input type="checkbox"/> Add 30 µl PS , mix well, incubate 5 min at RT.	
<input type="checkbox"/> Place on magnet for 2 - 5 min, discard supernatant.	
<input type="checkbox"/> Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.	
<input type="checkbox"/> Air dry beads for 5 - 10 minutes. ATTENTION: Do not let the beads dry too long!	
<input type="checkbox"/> Add 20 µl EB , remove from magnet, mix well, incubate 2 min at RT.	
<input type="checkbox"/> Place on magnet for 2 - 5 min, transfer 15 - 17 µl of the supernatant into a fresh PCR plate. <input type="checkbox"/> 🛑 Safe stopping point.	

6. Appendix A: RNA Input and PCR Cycles

Total RNA is the intended input for QuantSeq FWD and 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 (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 for QuantSeq FWD, and 10 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.18 and Appendix C, p.19).
- 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 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** (see Appendix D, p.20). 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.18).

** 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.20).

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.19 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 minutes).	
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.20). The qPCR assay should be performed also when RNA samples are of: <ul style="list-style-type: none">• 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 30 µl of Purification Beads (PB) for single-indexed libraries, or 35 µl for dual-indexed libraries.	Reduce volume of Purification Beads (PB) to 27 µl for single-indexed libraries, or 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:

- 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 (DV₂₀₀ of 51 %). The DV₂₀₀ is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV₂₀₀, the more degraded the RNA is.

ATTENTION:

- FFPE RNA samples are highly variable. Samples with lower mRNA content, or lower DV₂₀₀ values may require more PCR cycles. **We strongly recommend performing a qPCR assay** (using Lexogen's PCR Add-on Kit for Illumina (Cat. No. 020), Appendix D, p.20) 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 ~150 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 may be advisable 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: 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.17). **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. Enzyme Mix 3 (**E3** ●) and **PCR** ● supplied in the QuantSeq Kits, can be used interchangeably with **E** ● and **PCR** ● from the PCR Add-on Kit.

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

NOTE: SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines 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).

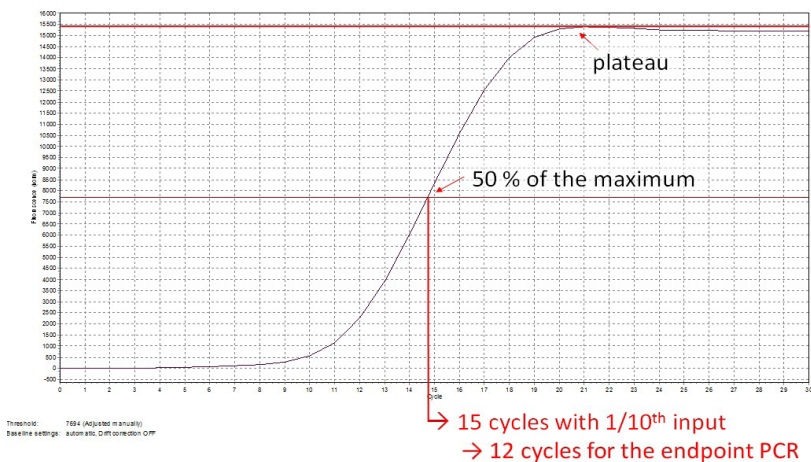


Figure 3. Calculation of the number of cycles for the endpoint PCR.

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 Single-Indexed Libraries (i7 only)

If your library yields are extremely low and insufficient for pooling, reamplification can be performed. Lexogen's PCR Add-on Kit contains a i7 Reamplification Primer (**i7-RE O**) that can be used to reamplify single-indexed (i7) libraries to get enough material for sequencing if they were undercycled. For details, please refer to the PCR Add-on Kit (Cat. No. 020) User Guide (020IM064). The Reamplification Add-on Kit for Illumina (080.96 - available upon request), can also be used to reamplify single-indexed libraries.

Reamplification of Dual-Indexed Libraries (i5 and i7)

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

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 C_q values to a set of known standards. While delivering a more accurate quantification, these assays do not supply the user with information regarding library size distribution. Unwanted side-products such as linker-linker artifacts are not discernible from the actual library in the qPCR assay as both will be amplified. Hence it is highly recommended to combine such an assay for quantification with microcapillary electrophoresis analysis for library size distribution.

If microcapillary electrophoresis platforms and qPCR machines are not available, very basic quality control can also be performed by separating a small aliquot of the library on a polyacrylamide or agarose gel. Library quantification can also be performed with an inexpensive benchtop fluorometer using one of several commercially available assays, e.g., Qubit dsDNA HS assay. Most UV-Vis spectrophotometers (e.g., NanoDrop, Thermo Fisher Scientific Inc.), are not sensitive enough to accurately quantify NGS libraries at these concentrations and should be avoided.

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 \geq 200 bp.

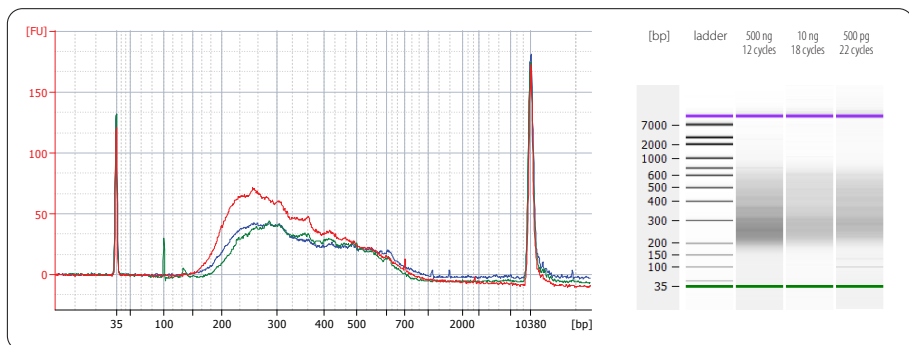


Figure 4. Bioanalyzer traces of QuantSeq FWD libraries prepared from different input amounts of total RNA input (UHRR). Libraries were prepared with the standard protocol, using 500 ng (red trace, 12 PCR cycles). Libraries with 10 ng (blue trace, 18 PCR cycles) and 500 pg (green trace, 22 PCR cycles) of UHRR input were prepared using low input protocol modifications (see Appendix B, p.18); 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).

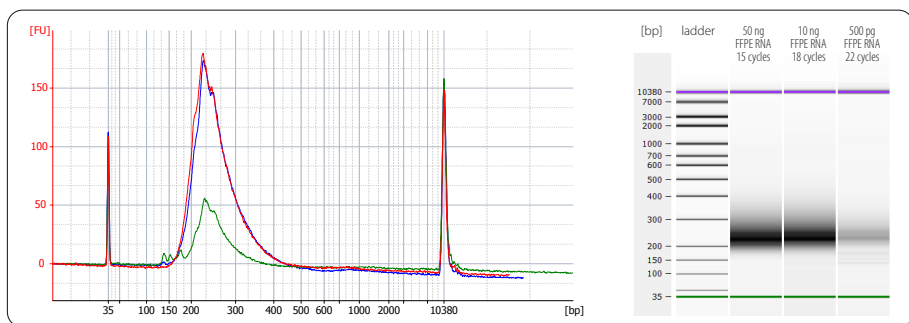


Figure 5. Bioanalyzer traces of QuantSeq FWD libraries synthesized from 50 ng (red trace), 10 ng (dark blue trace), and 500 pg (green trace), using mouse (Mm) brain FFPE RNA (RIN 1.8, DV₂₀₀ 51 %) as input. All libraries were prepared with the recommendations for FFPE RNA input (Appendix C, p.19). 500 pg FFPE RNA libraries already contain some artifacts below 150 bp which should be removed before sequencing, e.g., by repurifying the lane mix (see Appendix F, p.25).

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.20.

11. Appendix F: Multiplexing

QuantSeq libraries are designed for a high degree of multiplexing. The i7 indices represent the minimum requirement for multiplexed sequencing and are added during the PCR amplification. The i7 index primers are provided in 96-well plate format in all QuantSeq Library Prep Kits (Cat. No. 015, 016).

Single Indexing - i7 Indices

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

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

The Lexogen i7 6 nt index sequences are available for download at www.lexogen.com.

In general, we recommend processing a minimum of 8 samples, using a complete set of eight i7 indices for multiplexing (e.g., **7001-7008**). However, if fewer indices are required care should be taken to select indices that give a well-balanced signal in both lasers (red and green channels) for each nucleotide position. All columns (1 - 12) and rows (A - H) fulfill these criteria when individual libraries are mixed in an equimolar ratio. Use the online Index Balance Checker tool available at <https://www.lexogen.com/support-tools/index-balance-checker/>, to select the ideal combination of indices for optimal color and nucleotide balance.

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

Dual Indexing - Lexogen 6 nt i5 Indices

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

The Lexogen i5 6 nt Unique Dual Indexing Add-on Kit (5001–5096, Cat No. 047.96) provides a 96-well plate containing 96 unique i5 indices. This kit is designed for unique dual indexing in combination with Lexogen's 96 i7 indices (included in all QuantSeq FWD and REV Library Prep Kits). Up to 96 uniquely dual-indexed libraries can be prepared for sequencing in a single lane or run. Alternatively, used together with the 96 i7 indices, up to 9,216 dual-indexed libraries with different i5 / i7 index combinations can be multiplexed in a single sequencing lane or run.

The Lexogen i5 6 nt Dual Indexing Add-on Kit (5001–5004, Cat. No. 047.4) provides four different i5 index primers (5001–5004). Each tube contains sufficient volume for preparing 96 libraries (Cat. No. 047.4x96). In combination with 96 i7 indices, a maximum of 384 (4 i5 x 96 i7) dual-indexed libraries with different i5 / i7 index combinations can be multiplexed in a single sequencing lane or run.

Dual Indexing - Lexogen 12 nt Unique Dual Indexing Add-on Kits

QuantSeq Library Prep Kits (FWD and REV) are compatible with UDI 12 nt Unique Dual Indexing Add-on Kits (Cat. No. 107 - 111, 120). For details, please refer to the Lexogen 12 nt Unique Dual Indexing Add-on Kits User Guide (107UG223).

Additionally, QuantSeq FWD library prep kits are available as bundles with the 12 nt UDIs (Cat. No. 113 - 115 and 129 - 131). For details, please refer to the QuantSeq Library Prep Kit FWD with Unique Dual Indices (113UG227).

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 160 - 1,000 bp to include the whole library size distribution, and to exclude any linker-linker artifacts (150 bp when using 6nt i7 single-indexing), or overcycling bumps (>1,000 bp).

Molarity is then calculated from the average library size and the concentration (ng/μl) using the following equation:

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

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

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

Lane Mix Repurification to Remove Linker-Linker Artifacts

A shorter side-product representing linker-linker artifacts is sometimes visible at ~150 bp when using 6 nt i7 single-indexing, 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 (≤ 150 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 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.

QuantSeq FWD Libraries with i7 Indexing

i7 indices (6 nt) are introduced during PCR (step 27). For QuantSeq FWD libraries, Read 1 directly corresponds to the mRNA sequence.

```
5'-(Read 1 Sequencing Primer)-3'
5' AATGATACGGCGACCAACGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT- (Insert...
3' TTAATATGCGCTGGTGGCTCTAGATGTGAGAAAGGATGTGCTGCGAGAAGGCTAGA- (Insert...

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

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

5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

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

5' GATCGGAAGAGCACACGCTCTGAACTCCAGTCAC 3'

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

5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

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

ATTENTION: We do not recommend paired-end sequencing for QuantSeq FWD (Cat. No. 015) libraries, as the quality of Read 2 would be very low due to the poly(T) stretch at the beginning of Read 2. In case QuantSeq FWD libraries are sequenced in paired-end mode, Read 2 should be discarded and downstream data analysis should be performed using only Read 1. Read 1 read quality is not adversely affected in paired-end runs.

However, if paired-end sequencing for alignment of read pairs is required, please use QuantSeq REV (Cat. No. 016).

QuantSeq REV Libraries with i7 Indexing (Cat. No. 016)

i7 indices (6 nt) are introduced during PCR (step 27).

For QuantSeq REV libraries, Read 1 corresponds to the cDNA sequence.

```

5'-(Read 1 Custom Sequencing Primer)-3'
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTCCGATCT-TTTTTTTTTTTTTTTTTT-Insert...
3' TTAATAATGATACGGCGACCACCGAGATCTAGATGTGAGAAAGGGATGTGCTGCGAGAAAGGCTAGA-AAAAAAAAAAAAAAAAAA-Insert...

5'-(Index Read Sequencing Primer)-3'
...Insert- AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGCTCTCTGCTTG 3'
...Insert- TCTAGCCTTCTCTGCTGCAGACTTGAGGTCAGTG-i7-TAGAGCATAACGGCAGAACGACGAAC 5'
3'-(Read 2 Sequencing Primer)-5'

```

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

5' ACACTCTTTCCCTACACGACGCTCTCCGATCTTTTTTTTTTTTTTTTTTT 3'

ATTENTION: Do not use Multiplex Read 1 Sequencing Primer for QuantSeq REV (Cat. No. 016). Multiplex Read 1 Sequencing primer would result in a failed sequencing run as cluster calling would be impossible due to the poly(T) stretch.

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

5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

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

5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Usage of the Custom Sequencing Primer

For QuantSeq REV (Cat. No. 016) 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.

NOTE: QuantSeq REV kits were previously supplied a different version **CSP** ● (Version 2, prior to 2019) that is not compatible with all Illumina instruments. Please check the **CSP** ● version number on the tube provided before sequencing.

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.

HiSeq 2000, HiSeq 2500 (**CSP** ● added on cBot)

The **CSP** ● should be provided in a tube strip at 0.5 μM final concentration in a volume of 120 μl (final concentration 0.5 μM , to be diluted in HT1 = Hybridization buffer). Take 0.6 μl of 100 μM **CSP** ● and add 119.4 μl of HT1 buffer per sequencing lane. Place the 8-tube strip into the cBot position labeled primers.

HiSeq 2500 (**CSP** ● replaces HP10 in cBot Cluster Generation Reagent Plate)

Alternatively, the **CSP** ● can be placed directly into the cBot Cluster Generation Reagent Plate.

ATTENTION: The standard Illumina Multiplex Read 1 Sequencing Primer solution HP10 (for V4 chemistry located in row 2) provided in the cBot Cluster Generation Reagent Plate has to be **REMOVED** first! The Illumina V4 chemistry cBot Cluster Generation Reagent Plate only has 8 rows filled. A simple trick is to have the empty rows facing towards you, this way if you want to use a CSP in lane 1, you have to remove the HP10 solution from well 1 (first one on the far left) of the second row, rinse the well a couple of times with HT1 and then add the diluted **CSP** ●. For this take 1.25 μl of 100 μM **CSP** ● and add 248.75 μl of HT1 buffer per sequencing lane. The **CSP** ● should be at 0.5 μM final concentration in a volume of 250 μl (final concentration 0.5 μM , to be diluted in HT1 = Hybridization buffer). **ATTENTION:** Do not add the **CSP** ● to the Standard Illumina Multiplex Read 1 Sequencing Primer = HP10 solution! Always use fresh HT1 and add the **CSP** ● / HT1 dilution to the empty and rinsed well.

HiSeq 2500 - Rapid Run

Add 12.5 μl of 100 μM **CSP** ● to 2,487.5 μl HT1 = Hybridization buffer, resulting in a total volume of 2.5 ml and a final **CSP** ● concentration of 0.5 μM . In a rapid run, both lanes will use the same sequencing primer. It is not possible to run the two lanes with different sequencing primers.

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.

HiSeq 3000, HiSeq 4000 (CSP ● replaces HP10 in cBot Cluster Generation Reagent Plate)

The direct addition of custom sequencing primers is currently not supported on HiSeq 3000 and 4000 machines. However, the **CSP ●** can be used when performing cluster generation with the cBot Cluster Generation Reagent Plate. See the instructions above for the HiSeq 2500 (**CSP ● REPLACES** HP10 in the cBot Cluster Generation Reagent Plate). **ATTENTION:** Do not add the **CSP ●** to the HP10 solution! A primer mixture would result in low clusters calls and the resulting reads would be contaminated by poly(T) stretches. Always use fresh HT1 and add the **CSP ●** / HT1 dilution to the empty and rinsed well.

NextSeq

The **CSP ●** Version 5 is optimized for use on NextSeq 500 / 550 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. Please refer to page 7 of the NextSeq System Custom Primers Guide (Illumina) for further loading instructions.

ATTENTION: The **CSP ●** Version 2 included in QuantSeq REV kits produced prior to 2019 is not compatible with NextSeq instruments! Please check the **CSP ●** version number on the tube provided before sequencing.

MiniSeq and NovaSeq

Please contact support@lexogen.com if you wish to run QuantSeq REV 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.

QuantSeq is available in two read orientations: 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.

In QuantSeq REV (Cat. No. 016), 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 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. For paired-end sequencing where alignment of read pairs is required, we recommend using QuantSeq REV (Cat. No. 016).

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 6 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 ide-mux 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

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.

For QuantSeq REV data, single-read sequencing does not require any trimming using QuantSeq REV (Cat. No. 016). However, paired-end sequencing may require the first 12 nucleotides of Read 2 to be trimmed. Alternatively, also here the STAR aligner could be used with the number of allowed mismatches being set to 16 for paired-end reads.

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 and REV). The reads may not land in the last exon and span a junction hence splice-aware aligners should be used. Bowtie2, BMap, 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 (Cat. No. 016). 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 24 prep kit, 24 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 or REV-specific pipelines are automatically encoded in the allocated activation code supplied with the respective QuantSeq Kit, ensuring that the correct pipeline parameters are used for the different FWD and REV read orientations.

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 QuantSeq automation 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 widely, so if you are interested in QuantSeq automation 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 (<https://www.lexogen.com/quantseq-3mr-na-sequencing/#quantseqautomation>).

The standard QuantSeq 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 QuantSeq automation protocols, are available upon request. Please email support@lexogen.com for more information.

15. Appendix J: Revision History

The revision history table below shows User Guide versions and kit changes made from August 2018 onwards. Previous revision history is available from <https://www.lexogen.com/docs/>.

Publication No. / Revision Date	Change	Page
015UG009V0260 Aug. 16, 2021	Updated legal information.	2
	Removed General Guidelines.	8
	Updated QuantSeq-Flex information.	8
	Updated recommended input range to: 1 ng - 500 ng.	8 - 9, 17
	Updated references to Globin and BC1 Block solutions, and UMI Module for QuantSeq (removal of dedicated Appendices).	9 - 17
	Streamlined information in Appendices.	17 - 34
	Updated demultiplexing instructions.	32
015UG009V0252 Apr. 3, 2020	Updated General terms and conditions.	2
	Updated storage recommendations of EB buffer.	6
	Added references to Lexogen UDI 12 nt Unique Dual Indexing.	4, 15, 16, 36, 48
	Reformatted Step 29.	16
	Updated recommendations for DNase I treatment.	20
015UG009V0251 Feb. 26, 2019	Naming changes for: "i7 Index Plate" to "Lexogen i7 6 nt Index Set", and "i5 Dual Indexing Add-on Kit" to "Lexogen i5 6 nt Dual Indexing Add-on Kit".	6 - 48
	New CSP Version (V5) included in REV kits - CSPV5 is supported for use on HiSeq, MiSeq, MiniSeq, and NextSeq instruments.	39 - 41
	"REMARK" notes in steps 1 - 4 edited to refer only to specific instructions for low input/low quality/FFPE samples.	11 - 12
	Instructions for use of RS-Globin Block and UMI modules in place of RS and SS1, respectively, were added to detailed protocol steps 5 and 7.	12 - 13
	Reformatting of attention notes in detailed protocol.	11 - 17
	Short protocol format updated to include clear steps for low quality / low input / FFPE protocol modifications, and to include qPCR assay steps.	18 - 19
	Text and figures updated for Appendices A - N.	20 - 45
	Revision history table includes 2017 onwards only.	46-47
015UG009V0241 Aug. 27, 2018	Updated sequencing guidelines for Lexogen libraries.	39

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)
044 (Lexogen i7 6 nt Index Set (7001-7096))
047 (Lexogen i5 6 nt Dual Indexing Add-on Kits (5001-5096))
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)
113 - 115, 129 - 131 (QuantSeq 3' mRNA-Seq Library Prep Kit FWD with UDI 12 nt 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 · User Guide

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