

The background of the entire page is a light blue and white graphic. It features numerous translucent blue spheres of various sizes, some of which are connected by thin, light blue lines, creating a network-like or molecular structure. The spheres have highlights and shadows, giving them a three-dimensional appearance.

**LEXOGEN**

The RNA Experts

**QUANT<sup>TM</sup>**  
**SEQ**

Sequencing that counts

# 3' mRNA-Seq Library Prep Kit FWD HT User Guide

Catalog Numbers:

015 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (FWD) HT including i5 Dual Indexing Add-on Kit (5001-5004))

015UG110V0200

## **FOR RESEARCH USE ONLY. NOT INTENDED FOR DIAGNOSTIC OR THERAPEUTIC USE.**

INFORMATION IN THIS DOCUMENT IS SUBJECT TO CHANGE WITHOUT NOTICE.

Lexogen does not assume any responsibility for errors that may appear in this document.

## **PATENTS AND TRADEMARKS**

The QuantSeq 3' mRNA-Seq Library Prep Kits are covered by issued and/or pending patents. QuantSeq™ and SIRV™ are trademarks of Lexogen. The SIRVs are covered by issued and/or pending patents. Lexogen UDI 12 nt Unique Dual Index design and UDI sequences are covered by issued and/or pending patents. Lexogen is a registered trademark (EU, CH, US, CN, AU, NO, BR).

Illumina® is a registered trademark of Illumina, Inc.; RNasin® is a registered trademark of Promega Corporation. Bioanalyzer®, Fragment Analyzer™, and TapeStation® are trademarks of Agilent Technologies, Inc.; SYBR® Green I is a registered trademark of Molecular Probes, Inc.; LabChip® GX II is a registered trademark of Perkin Elmer. RNaseZap™, RNAlater®, and ERCC are registered trademarks of Ambion, Inc.; Thermomix® is a registered trademark of Eppendorf AG. Qubit™ and NanoDrop™ are registered trademarks of Thermo Fisher Scientific. All other brands and names contained in this user guide are the property of their respective owners. Lexogen does not assume responsibility for patent infringements or violations that may occur with the use of its products.

## **LIABILITY AND LIMITED USE LABEL LICENSE: FOR RESEARCH USE ONLY**

This document is proprietary to Lexogen. The QuantSeq kits are intended for use in research and development only. They need to be handled by qualified and experienced personnel to ensure safety and proper use. Lexogen does not assume liability for any damage caused by the improper use or the failure to read and explicitly follow this user guide. Furthermore, Lexogen does not assume warranty for merchantability or suitability of the product for a particular purpose.

The purchase of the product is subject to Lexogen general terms and conditions ([www.lexogen.com/terms-and-conditions/](http://www.lexogen.com/terms-and-conditions/)) and does not convey the rights to resell, distribute, further sub-license, repackage, or modify the product or any of its components. This document and its content shall not be used or distributed for any other purpose and/or otherwise communicated, disclosed, or reproduced in any way without the prior written consent of Lexogen.

For information on purchasing additional rights or a license for use other than research, please contact Lexogen.

## **WARRANTY**

Lexogen is committed to providing excellent products. Lexogen warrants that the product performs to the standards described in this user guide up to the expiration date. Should this product fail to meet these standards due to any reason other than misuse, improper handling, or storage, Lexogen will replace the product free of charge or issue a credit for the purchase price. Lexogen does not provide any warranty if product components are replaced with substitutes.

Under no circumstances shall the liability of this warranty exceed the purchase price of this product. We reserve the right to change, alter, or modify any product without notice to enhance its performance.

## **LITERATURE CITATION**

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

We reserve the right to change, alter, or modify any product without notice to enhance its performance.

## **CONTACT INFORMATION**

---

### **Lexogen GmbH**

Campus Vienna Biocenter 5  
1030 Vienna, Austria  
[www.lexogen.com](http://www.lexogen.com)  
Email: [support@lexogen.com](mailto:support@lexogen.com)

### **Support**

E-mail: [support@lexogen.com](mailto:support@lexogen.com)  
Tel. +43 (0) 1 3451212-41  
Fax. +43 (0) 1 3451212-99

# Table of Contents

1. Overview . . . . .	4
2. Kit Components and Storage Conditions . . . . .	6
3. User-Supplied Consumables and Equipment . . . . .	8
4. Detailed Protocol . . . . .	9
4.1 Library Generation . . . . .	9
4.2 Library Amplification . . . . .	13
4.2.1 Single Indexing (i7 only) . . . . .	14
4.2.2 Dual Indexing (i7 and i5) . . . . .	16
5. Short Procedure. . . . .	19
6. Appendix A: RNA Input and PCR Cycles . . . . .	21
7. Appendix B: Low Input RNA . . . . .	22
8. Appendix C: Low Quality RNA - FFPE. . . . .	23
9. Appendix D: qPCR and Library Reamplification . . . . .	25
10. Appendix E: Library Quality Control . . . . .	28
11. Appendix F: Multiplexing . . . . .	30
12. Appendix G: Sequencing* . . . . .	34
13. Appendix H: Data Analysis . . . . .	37
14. Appendix I: Automation . . . . .	39
15. Appendix J: Revision History . . . . .	40
16. Notes. . . . .	41

# 1. Overview

Lexogen's QuantSeq kit FWD HT provides a high-throughput library preparation protocol designed 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. The sequences obtained are close to the 3' end of the transcripts.

QuantSeq FWD HT (Cat. No. 015.384) is a high-throughput version (384 preps) with optional dual indexing to prevent sample mix-ups and to increase the number of available indices. The Read 1 linker sequence is located at 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 HT. Read 2 would start with the poly(T) stretch, and as a result of sequencing through the homopolymer stretch the quality of Read 2 would be very low.

QuantSeq FWD HT maintains strand-specificity and allows mapping of reads to their corresponding strand on the genome, enabling the discovery and quantification of antisense transcripts and overlapping genes. The kit includes magnetic beads for the purification steps and hence is compatible with automation. Multiplexing of libraries can be carried out using up to 96 i7 6 nt indices and 4 i5 6 nt indices (included in the kit). Additional i5 6 nt Dual Indexing Add-on Kits (Cat. No. 047) are also available for preparing and multiplexing up to 9,216 libraries. QuantSeq FWD HT is also compatible with Lexogen's 12 nt Unique Dual Indexing Add-on Kits (Cat. No. 107 - 111, 120). For convenience, a QuantSeq 3' FWD Library Prep Kit with 384 Unique Dual Indices (Set A) is also available (Cat. No. 115).

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.21. 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. 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. Each purchased QuantSeq kit provides a code for free data analysis, including differential expression (DE) analysis on the web-based pipeline service (for more details, please read Appendix H, p.37).

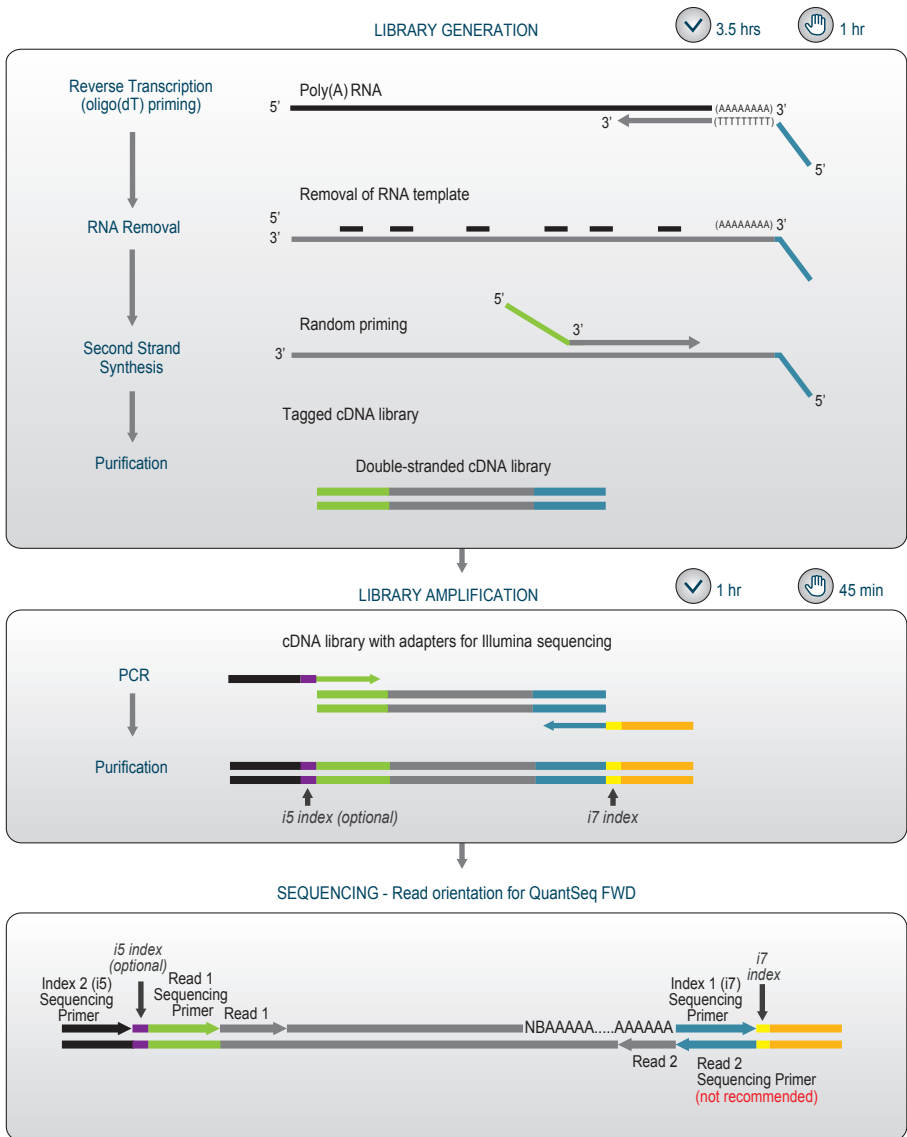


Figure 1. Schematic overview of the QuantSeq FWD HT library preparation workflow (Cat. No. 015.384). Illumina P5 adapters (Read 1) are shown in green and Illumina P7 adapters (Read 2) are shown in blue. Read 1 reflects the mRNA sequence. Paired-end sequencing is not recommended for QuantSeq FWD HT. *i7* and optional *i5* indices are introduced during the PCR amplification step and allow multiplexing of up to 384 samples in one sequencing lane or run.

## 2. Kit Components and Storage Conditions

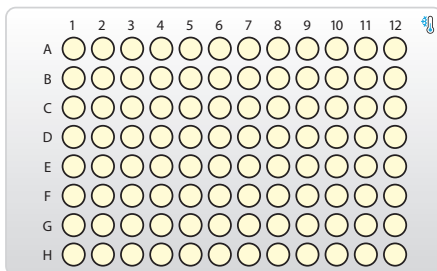
Library Generation Module (-20 °C)



i5 Dual Indexing Add-on Kit, Cat. No. 047.4x96 (-20 °C)



Lexogen i7 6 nt Index Set 7001-7096 (-20 °C)



Purification Module Cat. No. 022 (+4 °C)

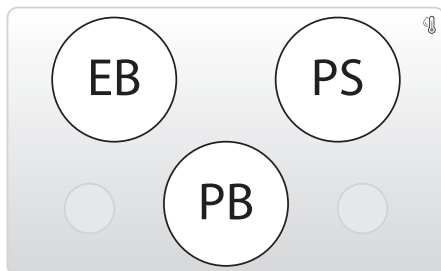




















Figure 2. Location of kit components. QuantSeq FWD HT Cat. No. 015.384 contains:

- one Library Generation Module (consisting of five different inlays, as depicted)
- one i5 Dual Indexing Add-on Kit (Cat. No. 047.4x96) composed of four perfectly balanced i5 indices (5001-5004) for optional dual indexing and two tubes of Dual PCR mastermix
- four Lexogen i7 6nt Index Sets, each plate with 96 single use indices (7001-7096) (only one is depicted)
- four Purification Modules (Cat. No. 022) for a total of 384 reactions (only one is depicted).

Kit Component	Tube Label	Volume* 384 preps	Storage
First Strand cDNA Synthesis Mix 1	FS1 ●	2x 1,056 µl	 -20 °C
First Strand cDNA Synthesis Mix 2	FS2 ●	4x 1,003 µl	 -20 °C
Enzyme Mix 1	E1 ●	2x 106 µl	 -20 °C
RNA Removal Solution	RS ○	2x 1,056 µl	 -20 °C
Second Strand Synthesis Mix 1	SS1 ●	4x 1,056 µl	 -20 °C
Second Strand Synthesis Mix 2	SS2 ●	2x 845 µl	 -20 °C
Enzyme Mix 2	E2 ●	2x 212 µl	 -20 °C
PCR Mix	PCR ●	2x 1,479 µl	 -20 °C
Enzyme Mix 3	E3 ●	2x 212 µl	 -20 °C
Lexogen i7 6 nt Index Set (96-well plates)		5 µl / reaction	 -20 °C
<b>i5 Dual Indexing Add-on Kit (Cat. No. 047.4x96) included in the kit</b>			
Dual PCR Mix	Dual PCR ●	2x 1,479 µl	 -20 °C
i5 Index 01	5001 ●	528 µl	 -20 °C
i5 Index 02	5002 ●	528 µl	 -20 °C
i5 Index 03	5003 ●	528 µl	 -20 °C
i5 Index 04	5004 ●	528 µl	 -20 °C
<b>Purification Modules (4x Cat. No. 022.96) included in the kit</b>			
Purification Beads	PB	4x 5,386 µl	 +4 °C
Purification Solution	PS	4x 9,082 µl	 +4 °C
Elution Buffer	EB	4x 13,411 µl	 +4 °C

\*including ≥10 % surplus

Upon receiving the QuantSeq kit, store the Purification Module (Cat. No. 022.96), 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.

### 3. User-Supplied Consumables and Equipment

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

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

#### Reagents / 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 Bio-analyzer).
- 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.23, for more information on RNA quality. Consult Appendix E, p.28 for information on library quantification methods.



# 4. Detailed Protocol

## 4.1 Library Generation

### Preparation

First Strand cDNA Synthesis	RNA Removal	Second Strand Synthesis	Purification
<b>FS1</b> ● – thawed at RT <b>FS2</b> ● – thawed at RT <b>E1</b> ● – keep on ice or at -20 °C	<b>RS</b> ○ – thawed at RT	<b>SS1</b> ● – thawed at 37 °C <b>SS2</b> ● – thawed at RT <b>E2</b> ● – keep on ice or at -20 °C	<b>PB</b> – stored at +4 °C <b>PS</b> – stored at +4 °C <b>80 % EtOH</b> – provided by user <b>prepare fresh!</b> <b>EB</b> – stored at +4 °C
85 °C, 3 min cool to 42 °C; 42 °C, 15 min	95 °C, 10 min (5 min if ≤1 ng) 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 HT is 1 ng. Input amounts ≤1 ng can be used, but please refer to Appendix B, p.22 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.22, and Appendix C, p.23).

**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 (166UG323). 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. In this case, 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.
- 

- 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.
- 4 **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:** After incubation, 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 O**). 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 O**) 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 (for 1 ng or less input, incubate for 5 minutes only), 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 HT libraries. The UMI Second Strand Synthesis Mix (**USS** ●) replaces the Second Strand Synthesis Mix 1 (**SS1** ●) from the standard QuantSeq FWD HT 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.
- 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.22 and Appendix C, p.23).
- 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

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

- For single indexing (i7 index only), follow 4.2.1 Single Indexing, p.14.
- For dual indexing (i5 and i7 indices), follow 4.2.2 Dual Indexing, p.16.

**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.25.

- 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.
- When preparing dual-indexed libraries, use the **Dual PCR Mix (Dual PCR ●)**. **DO NOT USE** the yellow-cap **PCR Mix (PCR ●)** as this contains a non-indexed P5 primer and will result in a loss of i5 indexing.
- The total PCR volumes differ for single indexing (30 µl) vs. dual indexing (35 µl) protocols.
- The volumes of Purification Beads (**PB**) added at step 29 for each protocol also differ accordingly:
  - 4.2.1 Single Indexing, p.14 - single indexing: add 30 µl **PB** for standard input or 27 µl **PB** for low input / low quality / FFPE samples.
  - 4.2.2 Dual Indexing, p.16 - dual indexing: add 35 µl **PB** for standard input or 31.5 µl **PB** for low input / low quality / FFPE samples.

### 4.2.1 Single Indexing (i7 only)

Single indexing PCR (i7 indices only) enables multiplexing and unique indexing of 96 libraries. i7 indices (**7001-7096**) are provided in the Lexogen i7 6 nt Index Set, in a 96-well plate format.

**NOTE:** The QuantSeq FWD HT Library Prep Kit is compatible with Lexogen's 12 nt Unique Dual Indexing Add-on Kits. For protocol details, please refer to the Lexogen 12 nt Unique Dual Indexing Add-on Kits User Guide (107UG223).

### Preparation

PCR	Purification
<b>PCR</b> ● – thawed at RT <b>E3</b> ● – keep on ice or at -20 °C <b>i7 6 nt Index Set</b> – thawed at RT; <b>spin down before opening!</b>	<b>PB</b> – stored at +4 °C <b>PS</b> – stored at +4 °C <b>80 % EtOH</b> – provided by user; <b>prepare fresh!</b> <b>EB</b> – 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.25	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.

**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.


**ATTENTION:** 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 12 - 26 cycles of PCR (as determined by qPCR, see Appendix D, p.25) with: Initial denaturation for 30 seconds at 98 °C; 12 - 26 cycles of 10 seconds at 98 °C, 20 seconds at 65 °C and 30 seconds at 72 °C, and a final extension for 1 minute at 72 °C, hold at 10 °C.  Safe stopping point. Libraries can be stored at -20 °C at this point.

## Purification

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

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

- 29 For **QuantSeq** libraries (**standard input >10 ng**), add 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.
- 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), 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.28), pooling (for multiplexing, Appendix F, p.30), and cluster generation.

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

## 4.2.2 Dual Indexing (i7 and i5)

The following protocol replaces the single indexing PCR and post-PCR purification protocol described in steps 25 - 41 on pages 14 - 16. If qPCR is intended to determine the exact cycle number for endpoint PCR, use the PCR Add-on Kit (Cat. No. 020, see also Appendix E, p.28).

**ATTENTION:** For dual indexing use the **Dual PCR Mix (Dual PCR ●)**. **DO NOT USE** the yellow-cap **PCR Mix (PCR ●)**, as this contains a non-indexed P5 primer and will result in a loss of i5 indexing.

PCR		Purification (Cat. No. 022)
<p><b>From i5 Dual Indexing Add-on Kit:</b></p> <p>Dual PCR ●</p> <p>5001 - 5004 ● or</p> <p>5001 - 5096 (i5 Index Plate, Cat. No. 047.96)</p> <p><b>From standard library prep kit:</b></p> <p>7001 - 7096 (i7 6 nt Index Set )</p> <p>E3 ●</p> <p>– thawed at RT</p> <p>– thawed at RT</p> <p>– thawed at RT</p> <p>– thawed at RT</p> <p>– keep on ice or at -20 °C</p> <p>} spin down before opening!</p>		<p><b>from standard library prep kits:</b></p> <p>PB – stored at +4 °C</p> <p>PS – stored at +4 °C</p> <p>80 % EtOH – provided by user</p> <p><b>prepare fresh!</b></p> <p>EB – stored at +4 °C</p>
Thermocycler	<p>98 °C, 30 sec</p> <p>98 °C, 10 sec</p> <p>65 °C, 20 sec</p> <p>72 °C, 30 sec</p> <p>72 °C, 1 min</p> <p>10 °C, ∞</p> <p>} 12- 26x</p> <p>see Appendix D, p.25</p>	<p>Equilibrate all reagents to room temperature for 30 minutes prior to use.</p>

## PCR

**NOTE:** At this point we recommend placing the Purification Module (**PB, PS, EB**) for step 30 at room temperature to give them enough time to equilibrate.

25

Prepare a mastermix containing 7 µl Dual PCR Mix (**Dual PCR ●**) and 1 µl Enzyme Mix 3 (**E3 ●**) per reaction, mix well by pipetting, spin down.

26

Add 8 µl of this **Dual PCR / E3** mastermix to 17 µl of the eluted library.

27


Add 5 µl of the respective i5 Index Primer (**5001 - 5004 ●**, in microtubes, or **5001 - 5096**, in 96-well plate from Cat. No. 047.96). **ATTENTION:** Spin down the i5 tubes / Index Plate before opening! Pierce or cut open the sealing foil of the wells containing the desired barcodes. Avoid cross contamination! Reseal opened wells of the barcode plate after use to prevent cross contamination!



---

Add 5 µl of the respective i7 Index Primer (**7001 - 7096**, in 96-well plate). **REMARK:** Ensure the total PCR volume is 35 µl. If necessary, adjust the volume with Elution Buffer (**EB**) or molecular biology-grade water. Mix well by pipetting. Seal the PCR plate and quickly spin down. **ATTENTION:** Reseal opened wells of the barcode plate after use, to prevent cross contamination!

---

Conduct 12 - 26 cycles of PCR (as determined by qPCR, see Appendix D, p.25) with: Initial denaturation for 30 seconds at 98 °C, 12 - 26 cycles of 10 seconds at 98 °C, 20 seconds at 65 °C and 30 seconds at 72 °C, and a final extension for 1 minute at 72 °C, hold at 10 °C.  Safe stopping point. Libraries can be stored at -20 °C at this point.

---

## Purification

The finished library is purified from PCR components that can interfere with quantification. The Purification Beads (**PB**) may have settled and must be properly resuspended before adding them to the reaction.

**ATTENTION:** If PCR products were stored at -20 °C, ensure these are thawed and equilibrated to room temperature before Purification Beads (**PB**) are added.

---

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.

---

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

---

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

---

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.

---

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.

---

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

---

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

---

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.

---

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

---

39

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.

40


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.

41

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

42

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.28), pooling (for multiplexing, Appendix F, p.30), 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 ( $\leq 10$ ng) / Low Quality / FFPE	Low Input ( $\leq 1$ ng)
<b>First Strand cDNA Synthesis</b>		
<input type="checkbox"/> Mix 5 $\mu$ l RNA and 5 $\mu$ l <b>FS1</b> ●.	<input type="checkbox"/> Skip!	
<input type="checkbox"/> Incubate for 3 min at 85 °C, then cool to 42 °C. <b>Keep samples on thermocycler at 42 °C!</b>	<input type="checkbox"/> Skip!	
<input type="checkbox"/> Prepare a mastermix with 9.5 $\mu$ l <b>FS2</b> ● and 0.5 $\mu$ l <b>E1</b> ● per reaction, mix well and pre-warm for 2 - 3 min at 42 °C.	<input type="checkbox"/> Prepare a mastermix with 5 $\mu$ l <b>FS1</b> ●, 9.5 $\mu$ l <b>FS2</b> ●, and 0.5 $\mu$ l <b>E1</b> ● per reaction, mix well and pre-warm for 2 - 3 min at 42 °C. <b>Place RNA samples at RT for 2 - 3 min.</b>	
<input type="checkbox"/> Add 10 $\mu$ l <b>FS2</b> / <b>E1</b> mix per reaction, mix well, and spin down. <b>Keep samples on thermocycler at 42 °C when adding mastermix!</b>	<input type="checkbox"/> Add 15 $\mu$ l <b>FS1</b> / <b>FS2</b> / <b>E1</b> mix to each 5 $\mu$ l RNA sample, mix well and spin down. <b>Transfer samples to thermocycler at 42 °C!</b>	
<input type="checkbox"/> Incubate for 15 min at 42 °C. <b>Proceed immediately to RNA Removal!</b>	<input type="checkbox"/> Incubate for 15 min <b>or</b> 1 hr at 42 °C. <b>Proceed immediately to RNA Removal!</b>	
<b>RNA Removal</b>		
<input type="checkbox"/> Add 5 $\mu$ l <b>RS</b> ○, mix well.		
<input type="checkbox"/> Incubate 10 min at 95 °C, cool to 25 °C.	<input type="checkbox"/> 10 min at 95 °C, cool to 25 °C	<input type="checkbox"/> 5 min at 95 °C, cool to 25 °C
<b>Second Strand Synthesis</b>		
<input type="checkbox"/> Add 10 $\mu$ l <b>SS1</b> ●, 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 $\mu$ l <b>SS2</b> ● and 1 $\mu$ l <b>E2</b> ● per reaction, mix well.		
<input type="checkbox"/> Add 5 $\mu$ l <b>SS2</b> / <b>E2</b> mix per reaction, mix well.		
<input type="checkbox"/> Incubate 15 min at 25 °C.  Safe stopping point.		
<b>Purification</b>		
<input type="checkbox"/> Add 16 $\mu$ l <b>PB</b> 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 $\mu$ l <b>EB</b> , remove from magnet, mix well, incubate 2 min at RT.		
<input type="checkbox"/> Add 56 $\mu$ l <b>PS</b> , mix well, incubate 5 min at RT.	<input type="checkbox"/> For low input / low quality / FFPE: Add 48 $\mu$ l <b>PS</b> , mix well, incubate 5 min at RT.	
<input type="checkbox"/> Place on magnet for 2 - 5 min, discard supernatant.		
<input type="checkbox"/> <input type="checkbox"/> Rinse beads twice with 120 $\mu$ l 80 % EtOH, 30 sec.		
<input type="checkbox"/> Air dry beads for 5 - 10 min. <b>ATTENTION:</b> Do not let the beads dry too long!		
<input type="checkbox"/> Add 20 $\mu$ l <b>EB</b> , remove from magnet, mix well, incubate 2 min at RT.		
<input type="checkbox"/> Place on magnet for 2 - 5 min, transfer 17 $\mu$ 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 and ≤1 ng) / Low Quality / FFPE
<b>qPCR [Strongly Recommended! Requires PCR Add-on Kit (Cat. No. 020.96)]</b>	
<input type="checkbox"/> Add 2 µl of <b>EB</b> 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 <b>PCR</b> ●, 5 µl Primer <b>7000</b> , 1 µl <b>E</b> ● (from PCR Add-on Kit), 1.2 µl of 2.5x SYBR Green I nucleic acid stain, and 14.1 µl of <b>EB</b> , per reaction. Mix well.	
<input type="checkbox"/> PCR: 98 °C, 30 sec. <div style="display: flex; align-items: center;"> <div style="margin-right: 10px;">           98 °C, 10 sec            65 °C, 20 sec            72 °C, 30 sec         </div> <div style="font-size: 3em; margin-right: 10px;">}</div> <div> <b>35x</b> </div> </div> 72 °C, 1 min 10 °C, ∞. Calculate the optimal cycle number for Endpoint PCR (see Appendix D, p.25).	
Single Indexing - i7 only	Dual Indexing - i5 and i7 Indices
<b>Endpoint PCR</b>	
<input type="checkbox"/> Prepare a mastermix with 7 µl <b>PCR</b> ● and 1 µl <b>E3</b> ● per reaction, mix well.	<input type="checkbox"/> Prepare a mastermix with 7 µl <b>Dual PCR</b> ● and 1 µl <b>E3</b> ● per reaction, mix well.
<input type="checkbox"/> Add 8 µl <b>PCR / E3</b> mastermix to 17 µl of each purified library.	<input type="checkbox"/> Add 8 µl <b>Dual PCR / E3</b> mastermix to 17 µl of each purified library.
<input type="checkbox"/> <b>Do not add i5 index primers!</b>	<input type="checkbox"/> Add 5 µl i5 Primer ( <b>5001-5004</b> ●) to each reaction, mix well.
<input type="checkbox"/> Add 5 µl i7 Primer ( <b>7001-7096</b> , from the 96-well plate) to each reaction, mix well. <b>ATTENTION:</b> Reseal opened index wells after usage!	
<input type="checkbox"/> PCR: 98 °C, 30 sec. <div style="display: flex; align-items: center;"> <div style="margin-right: 10px;">           98 °C, 10 sec            65 °C, 20 sec            72 °C, 30 sec         </div> <div style="font-size: 3em; margin-right: 10px;">}</div> <div> <b>12 - 26x</b> </div> </div> 72 °C, 1 min 10 °C, ∞,  Safe stopping point.	
<b>ATTENTION:</b> Increased cycle numbers may be required for low input / low quality / FFPE RNA. (see Appendix B, p.22 and Appendix C, p.23)	
<b>Purification</b>	
<input type="checkbox"/> Add 30 µl <b>PB</b> per reaction (or 35 µl for dual-indexed libraries), mix well, incubate 5 min at RT.	<input type="checkbox"/> For low input / low quality / FFPE: Add 27 µl <b>PB</b> per reaction (or 31.5 µl for dual-indexed libraries), 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 <b>EB</b> , remove from magnet, mix well, incubate 2 min at RT.	
<input type="checkbox"/> Add 30 µl <b>PS</b> , 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. <b>ATTENTION:</b> Do not let the beads dry too long!	
<input type="checkbox"/> Add 20 µl <b>EB</b> , 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. Safe stopping point.	

# 6. Appendix A: RNA Input and PCR Cycles

Total RNA is the intended input for QuantSeq FWD HT. 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  $\geq 200$  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 ( $\leq 10$  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.22 and Appendix C, p.23).
- The minimum recommended input for QuantSeq FWD HT 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.25). 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*	22 - 26
10 ng*	18 - 21
100 ng	15 - 18
$\geq 500$ ng	12 - 15

\* Using low input protocol modifications with 1 hour incubation at 42 °C at step 4 (See Appendix B, p.22).

\*\* 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.25).

## 7. Appendix B: Low Input RNA

Protocol modifications apply when using low input RNA ( $\leq 10$  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.23 for specific information on protocol recommendations for FFPE samples.

Working with input amounts  $\leq 1$  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 <b>FS1</b> to RNA samples. Do not place samples back on ice after adding <b>FS1</b> !	<b>Skip denaturation step!</b> Place RNA samples briefly at room temperature while the mastermix is prepared ( <b>2-3 min</b> ).	
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 <b>FS2 / E1</b> mastermix – pre-warm for 2 - 3 minutes at 42 °C.	Prepare <b>FS1 / FS2 / E1</b> mastermix – pre-warm for 2 - 3 minutes at 42 °C.	
Step 4	Add pre-warmed mastermix to <b>RNA / FS1</b> 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 ( <b>PS</b> ).	Reduce volume of Purification Solution ( <b>PS</b> ) 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.25). The qPCR assay should be performed also when RNA samples are of: <ul style="list-style-type: none"><li>• Variable input amount</li><li>• Variable quality (RIN / RQN) or purity (absorbance ratios: 260 / 280 and 260 / 230)</li><li>• Variable type (e.g., species, tissue, cell type)</li><li>• FFPE origin; or highly degraded</li></ul>		
Step 29	<b>Single-Indexed libraries:</b> Add 30 µl of Purification Beads ( <b>PB</b> ).	<b>Single-Indexed libraries:</b> Reduce volume of Purification Beads ( <b>PB</b> ) to 27 µl..	
Step 30	<b>Dual-Indexed libraries:</b> Add 35 µl of Purification Beads ( <b>PB</b> ).	<b>Dual-Indexed libraries:</b> Reduce volume of Purification Beads ( <b>PB</b> ) to 31.5 µl..	

## 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 (DV<sub>200</sub> of 51 %). The DV<sub>200</sub> is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV<sub>200</sub>, the more degraded the RNA is.

### ATTENTION:

- FFPE RNA samples are highly variable. Samples with lower mRNA content, or lower DV<sub>200</sub> 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.25) 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.28). 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.

- Mitochondrial ribosomal RNAs (mt-rRNAs) are polyadenylated and hence will also be reverse transcribed and converted into a cDNA library if oligo(dT) priming is used during First Strand Synthesis. mt-rRNAs can therefore be observed, but will only represent a minor fraction of the reads when using a 3' mRNA Seq protocol.
- Optionally, an rRNA depletion method, which also removes mt-rRNAs, such as Lexogen's Ribo-Cop rRNA Depletion Kit (144.24 and 144.96) can be used before starting the QuantSeq library preparation.
- If adding spike-in controls (e.g., SIRV-Set 3, Cat. No. 051), these should be added prior to any DNase I treatment. For further questions, please contact [support@lexogen.com](mailto: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.21). **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.28).

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 for 30 seconds at 98 °C, 35 cycles of 10 seconds at 98 °C, 20 seconds at 65 °C and 30 seconds at 72 °C, and a final extension for 1 minute at 72 °C, 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).

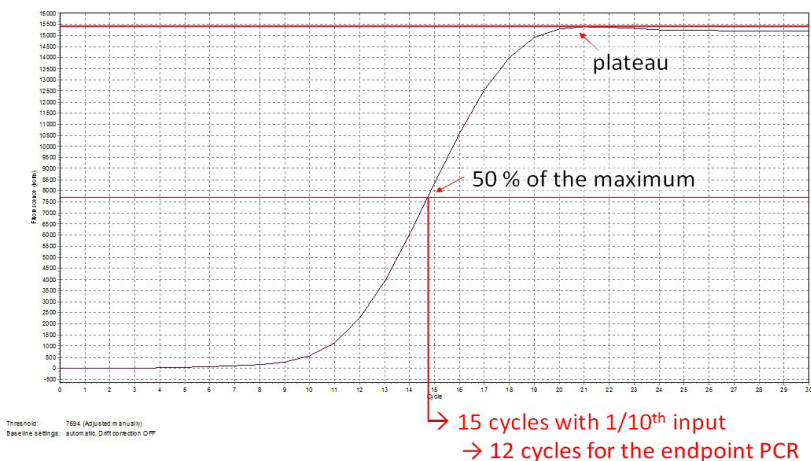


Figure 3. Calculation of the number of cycles for the endpoint PCR.

**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 (020UG064). 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](mailto:support@lexogen.com) for more information.

## 10. Appendix E: Library Quality Control

Quality control of finished QuantSeq HT 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  $\mu$ 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  $\mu$ l of the finished library may be diluted to the required volume (e.g., 2  $\mu$ l sample for TapeStation and 10  $\mu$ 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<sub>q</sub> 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.

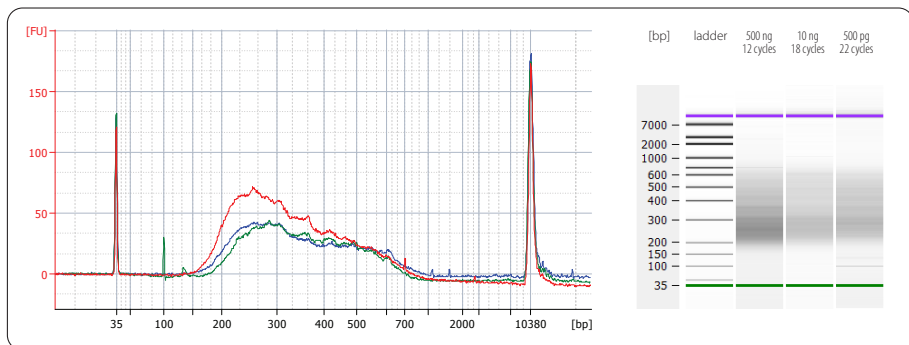


Figure 4. Bioanalyzer traces of QuantSeq FWD libraries prepared from different input amounts of total RNA input (UHRR). Libraries were prepared with the standard protocol, using 500 ng (red trace, 12 PCR cycles). Libraries with 10 ng (blue trace, 18 PCR cycles) and 500 pg (green trace, 22 PCR cycles)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

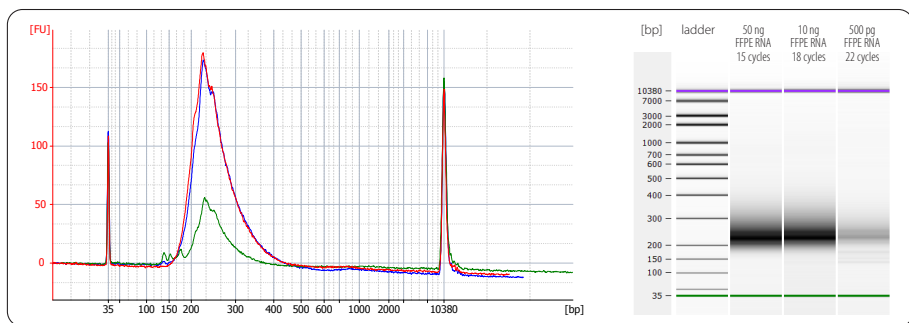


Figure 5. Bioanalyzer traces of QuantSeq FWD libraries synthesized from 50 ng (red trace), 10 ng (dark blue trace), and 500 pg (green trace), using mouse (Mm) brain FFPE RNA (RIN 1.8, DV<sub>200</sub> 51 %) as input. All libraries were prepared with the recommendations for FFPE RNA input (Appendix C, p.23). 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.30).

## 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.25.

# 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](http://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 - i5 and i7 6 nt Indices

Each of the four i5 6 nt indices provided in the QuantSeq 3' mRNA-Seq Kit FWD HT can be combined with any of the Lexogen i7 6 nt indices provided in the Lexogen i7 6 nt Index Set (**7001-7096**, in 96-well plate). By combining the four different i5 6 nt indices with the 96 different i7 6 nt indices, 384 different index combinations can be achieved.

Using the full set of 96 Lexogen i5 6 nt indices provided in the Lexogen i5 6 nt Unique Dual Indexing Add-on Kit (Cat. No. 047.96), together with the 96 Lexogen i7 6 nt indices provided in Lexogen's i7 6 nt Index Set (**7001-7096**, in 96-well plate) , a maximum of 9,216 different i5 / i7 index combinations can be achieved. Alternatively, up to 96 uniquely dual-indexed libraries can be multiplexed for sequencing without repeating the use of any of the i5 or i7 indices.

QuantSeq Library Prep Kits (FWD and REV) are compatible with 12 nt UDI Unique Dual Indexing Add-on Kits. For details, please refer to the Lexogen 12 nt Unique Dual Indexing Add-on Kits User Guide (107UG223) and the QuantSeq 3' mRNA-Seq Library Prep Kit FWD with Unique Dual Indices User Guide (113UG227).

## i5 6 nt Indices

The QuantSeq 3' mRNA-Seq Library Prep Kit FWD HT (Cat. No. 015.384) includes 4 perfectly balanced i5 6 nt indices (**5001-5004** ●, see Table below). Dual-indexed libraries prepared with these i5 indices are suitable for sequencing on all Illumina instruments listed below. Each nucleotide (A, C, G, and T) is present at each position of the 6 nt long index, resulting in perfect nucleotide and color balance.

Index 2 (i5) Oligo	i5 Sequences for Sample Sheet Entry	
	Forward Orientation (i5) MiSeq®, HiSeq® 2000/2500 (SR/PE), HiSeq® 3000/4000 (SR), NovaSeq™ 6000 (v1.0 reagents)	Reverse Complement Orientation (i5rc) MiniSeq™, NextSeq™, HiSeq® 3000/4000 (PE), iSeq 100, NovaSeq™ 6000 (v1.5 reagents)
<b>5001</b> ●	CGCCAT (AC)	ATGGCG (GT)
<b>5002</b> ●	ATTTTA (AC)	TAAAT (GT)
<b>5003</b> ●	GCAACG (AC)	CGTTGC (GT)
<b>5004</b> ●	TAGGGC (AC)	GCCCTA (GT)

Nucleotides in brackets (AC) or (GT) are derived from the Illumina adapters and are not actually part of the index. They are listed only for completeness if the sample sheet requires 8 nt to be entered.

Depending on the instrument workflow and flow cell type (paired-end, PE; single-read, SR), i5 indices are sequenced in either forward or reverse complement.

The following Illumina instruments read the i5 index in **forward orientation**:

- HiSeq® 2000/2500 (all SR and PE flow cells)
- HiSeq® 3000/4000 (SR flow cells only)
- MiSeq® (all (PE) flow cells)

- NovaSeq™ 6000 (v1.0 reagents, PE flow cells)

For the forward orientation read-out, the Index 2 Read (i5) is primed using the Grafted P5 Oligo on the flow cell (or the Index 2 (i5) Sequencing Primer for SR HiSeq® 2000/2500 flow cells). Therefore, the i5 index sequences must be entered into the sample sheet in the **forward** orientation (see Table p.29). If an 8-cycle index read length is used, the next two nucleotides of the downstream adapter sequence (i.e., AC) should be added to the end of the index sequence. For example, 5001 becomes CGCCAT**AC**.

The following Illumina instruments read the i5 index in **reverse complement** orientation:

- MiniSeq™ (all (PE) flow cells)
- NextSeq® 500/550/2000 (all (PE) flow cells)
- HiSeq® 3000/4000 (PE flow cells only)
- iSeq 100 (PE flow cell)
- NovaSeq™ 6000 (v1.5 reagents, PE flow cells)

For the reverse complement orientation read-out, the Index 2 Read (i5) is performed after Read 2 Resynthesis, using the Index 2 (i5) Sequencing Primer (see also Appendix G, p.34). Therefore, the i5 index sequences must be entered into the sample sheet in the **reverse complement** orientation (i5rc). If an 8-cycle index read length is used, the next two nucleotides of the downstream adapter sequence, (i.e., GT), should be added to the end of the index sequence. For example, 5001 becomes ATGGCG**GT**.

A file containing the complete set of forward and reverse complement i5 6 nt index sequences is available for download from the QuantSeq FWD Webpage via: <https://www.lexogen.com/quantseq-3mrna-sequencing/#quantseqdownloads>. Detailed workflows for dual indexed sequencing are provided in Appendix G, p.34.

## Index Balance

In general, it is important that each nucleotide (A, C, G, and T) is present at each position of the index reads (Index 1 Read, i7; and Index 2 Read, i5), and that the signal intensity of each nucleotide is perfectly balanced to maintain optimal base calling accuracy and read quality. This is particularly critical for instruments that use two-channel detection (e.g., NextSeq®, MiniSeq™, and NovaSeq™), in which: T is labeled with a green fluorophore, C is labeled with red, half of A is labeled red and the other half is labeled green, and G is unlabeled. The i5 indices 5001 - 5004 contain perfect nucleotide balance at each position of the i5 index read.

The index balance should be checked for both the i5 and i7 index sets separately when designing the multiplexing strategy for dual-indexed libraries. To determine the optimal balance for each index read (i5 and i7) for the specific number of libraries to be sequenced, we recommend using Lexogen's online Index Balance Checker tool, available at [www.lexogen.com/support-tools/index-balance-checker/](http://www.lexogen.com/support-tools/index-balance-checker/).



**NOTE:** Individual libraries within a lane or run should always be pooled at an equimolar ratio to preserve perfect nucleotide balance at each position of the index read. If non-equimolar library pooling is required, the relative molarity of each library can be adjusted using the Index Balance Checker tool, when selecting indices to use for your libraries.

## 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.).

---

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 6 nt i7 single-indexing, 160 bp when using 6 nt i5 / i7 dual-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](http://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<sub>2</sub>O).

---

**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.15).

---

## 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).

**ATTENTION:** We do not recommend paired-end sequencing for QuantSeq FWD HT (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 HT 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.

### 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

Dual indexing can be performed on single-read (SR) and paired-end (PE) flow cells. The workflow for dual-indexed library sequencing differs, depending on the Illumina instrument and flow cell type (see Table below).

Illumina Instruments	Flow Cell Type	No. Chemistry-Only Cycles <sup>2</sup>	Workflow <sup>1</sup>
HiSeq 2000 / 2500 HiSeq 3000 / 4000	SR	0	Forward Strand (A)
HiSeq 2000 / 2500 MiSeq NovaSeq 6000 (v1.0 reagent kits)	PE	7	
iSeq 100 MiniSeq NextSeq 500 - 2000 HiSeq 3000 / 4000 NovaSeq 6000 (v1.5 reagent kits)	PE	0	Reverse Complement (B)

<sup>1</sup>Workflows correspond to Illumina's Dual-Indexed Workflows.

<sup>2</sup>Additional chemistry-only (no-imaging) cycles are performed before the i5 index is read-out.

## QuantSeq FWD HT Libraries with dual indexing

i7 indices (6 nt) are introduced during PCR (step 27). For QuantSeq FWD libraries, Read 1 directly corresponds to the mRNA sequence. The Index 2 (i5) Read is performed in a different order with respect to Read 2 resynthesis depending on the dual indexing workflow of the instrument. The forward strand workflow reads the Index 2 Read (i5) before Read 2 resynthesis. The reverse complement workflow reads Index 2 Read (i5) after Read 2 resynthesis. Different instruments use different primers for index 2 read-out. Examples of forward and reverse complement i5 index read-out are provided below.

For more details about dual indexed library sequencing workflows for specific instruments, please refer to the Illumina Indexed Sequencing Overview resource, the Lexogen i5 6 nt Dual Indexing Add-on Kit Instruction manual (047UG109), the UDI 12 nt Unique Dual Indexing online FAQs, or contact [support@lexogen.com](mailto:support@lexogen.com).

## Forward Orientation i5 Read-Out - Index 2 Sequencing Primer

```

5'-(Index 2 (i5) Sequencing Primer)-3'      5'-(Read 1 Sequencing Primer)-3'
5'-AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert...
3'-TTACTATGCGCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert...

5'-(Index 1 (i7) Sequencing Primer)-3'
...Insert)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGCGAGAAGACGAAC 5'

```

## Forward Orientation i5 Read-Out - Grafted P5 Oligo

```

5'-(Grafted P5 Oligo)-3'      5'-(Read 1 Sequencing Primer)-3'
5'-AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert...
3'-TTACTATGCGCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert...

5'-(Index 1 (i7) Sequencing Primer)-3'

```

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

```

...Insert)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCGCTCTTCTGCTTG 3'
...Insert)-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'

```

## Reverse Complement Orientation i5 Read-Out

```

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

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

```

## Sequencing Primers

Standard Illumina sequencing primers are compatible with all QuantSeq dual-indexed libraries and are included in the flow cell reagent kits for the specified instrument. The sequences below are provided as a guideline only.

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

5'ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

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

5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

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

5'GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

**Index 2 Read (i5):**

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

5'AATGATACGGCGACCACCGAGATCTACAC 3'

Grafted P5 Oligo on Flow Cell (not supplied):

5'AATGATACGGCGACCACCGAGA 3'

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

5'AGATCGGAAGAGCGTCGTAGGGAAAGAGTGT 3'

# 13. Appendix H: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of QuantSeq FWD HT data and is kept as general as possible for integration with your standard pipeline.

QuantSeq is available in two read orientations: QuantSeq FWD HT (Cat. No. 015.384) 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/quantseq-data-analysis](http://www.lexogen.com/quantseq-data-analysis) and [www.lexogen.com/quantseq-data-analysis-rev](http://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](http://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](mailto: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 FWD data. The reads may not land in the last exon and span a junction hence splice-aware aligners should be used. Bowtie2, BBDMap, 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 on the web-based data analysis platform

With each purchased QuantSeq kit, you receive a code for free data analysis including differential expression (DE) analysis on the web-based data analysis platform (<https://www.lexogen.com/data-analysis-solutions/>). The activation code can be found on a label located on the cardboard above the microtube holder (or one of the holders, if the kit contains more than one). Each provided code allows for the same number of data analysis pipeline runs as the number of reactions included in the library prep kit (i.e., for a 96 prep kit, 96 analysis runs can be performed). The technical parameters used for QuantSeq data analysis pipelines on the web-based data analysis platform are available online at <https://www.lexogen.com/lexogen-data-analysis-solutions>.

Activation codes for additional pipeline runs can also be purchased from Lexogen. For further inquiries, please contact us at [support@lexogen.com](mailto:support@lexogen.com).

# 14. Appendix I: Automation

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

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

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

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

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

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

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

# 15. Appendix J: Revision History

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

Publication No./ Revision date	Change	Page
<b>015UG110V0200</b> Jan. 25, 2023	Updated legal information.	2
	Removed General Guidelines.	3
	Updated Kit Components Figure 2 and Table to reflect current packaging and storage requirements.	6-7
	Updated QuantSeq-Flex information and references to QuantSeq add-on modules (Globin and BC1 Block solutions, and UMI Module for QuantSeq - removal of dedicated Appendices).	4 - 39
	Updated recommended input range to: 1 ng - 500 ng.	10 -46
	Short procedure reformatting.	31 -32
	Sequencing Appendix G dual indexing workflow information updated.	46
	Streamlined information in Appendices.	6-48
	Updated demultiplexing instructions.	39-41
	Amended information about analysis pipeline (BlueBee®).	2, 4, 37, 40
	Appendix B (input amount and PCR cycle) tables updated.	12-13
	Low input protocol modification table Appendix C updated.	11-17
	Typical results library traces updated.	18-19
	Amended information about automation.	39
<b>015UG110V0111</b> Sep. 07, 2018	Updated sequencing guidelines for Lexogen libraries.	41
<b>015UG110V0110</b> Jun. 14, 2018	PCR Mix was renamed to Dual PCR Mix (no change to buffer composition) and kit component figure updated.	6, 17-18
	Added note to pre-warm the FS2 / E1 mastermix for 2 -3 minutes at 42 °C at step 2.	11
	Safe stopping point removed after step 4: Proceed immediately to RNA removal.	12
	Updated text for Appendices B and C and added table of protocol modifications for low input.	24-26
	Modified format and text for Appendix E: qPCR, and F: Library Reamplification.	27-28
	Added new Appendices I: Globin Block Modules, and J: Unique Molecular Identifiers.	34-37
	Updated information on dual indexing, multiplexing, and sequencing workflows in Appendices K and L.	38-44
	Reduced Revision History table to show updates from 2016 onwards.	47



# 16. Notes

## Associated Products:

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)  
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))  
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

Lexogen GmbH  
Campus Vienna Biocenter 5  
1030 Vienna, Austria  
Telephone: +43 (0) 1 345 1212-41  
Fax: +43 (0) 1 345 1212-99  
E-mail: [support@lexogen.com](mailto:support@lexogen.com)  
© Lexogen GmbH, 2023

Lexogen, Inc.  
51 Autumn Pond Park  
Greenland, NH 03840, USA  
Telephone: +1-603-431-4300  
Fax: +1-603-431-4333  
[www.lexogen.com](http://www.lexogen.com)