LEXOGEN
The RNA Experts

QUANT™ SEQ
Sequencing that counts

QuantSeq-Flex Second Strand Synthesis Module V2

User Guide

Catalog Number:
028 (QuantSeq-Flex Second Strand Synthesis Module V2 for Illumina)

028UG350V0100

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™ is a trademark of Lexogen. Lexogen is a registered trademark (EU, CH, USA). Lexogen UDI 12 nt Unique Dual Index design and UDI sequences are covered by issued and/or pending patents.
Illumina® is a registered trademark of Illumina, Inc. Bioanalyzer® is a trademark of Agilent Technologies, Inc. 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/) 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
For any publication using this product, please refer to it as Lexogen's QuantSeq-Flex Targeted RNA-Seq Library Prep Kit with Second Strand Synthesis Module V2.

CONTACT INFORMATION

Lexogen GmbH
Campus Vienna Biocenter 5
1030 Vienna, Austria
www.lexogen.com
E-mail: info@lexogen.com

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

Table of Contents

LEXOGEN

The RNA Experts

1. Overview

The QuantSeq-Flex Targeted Second Strand Synthesis Module is an Add-on Module that can only be used together with the QuantSeq FWD Library prep kit (Cat. No. 015 for single indexing or Cat. No. 113 - 115, 129 - 131 for Unique Dual Indexing).

This User Guide describes the usage of the QuantSeq-Flex Targeted Second Strand Synthesis Module only. The module should be used with oligo(dT) primed reverse transcription provided in the QuantSeq FWD Library prep kits.
For dual-targeted QuantSeq-Flex (targeted reverse transcription and targeted second strand synthesis), please contact support@lexogen.com.

The QuantSeq-Flex Targeted Second Strand Synthesis Module enables target-specific priming during second strand synthesis.
Reverse transcription is primed with an oligo(dT) primer (included in the QuantSeq FWD library prep kits) and target-specific primers (to be provided by the user) are added separately to the second strand synthesis reaction. When designing targeted primers, please be advised to include the Illumina P5 sequence (see Appendix A, p.10) at the 5' end of your second strand synthesis primers.

Targeted second strand synthesis primers enable a more streamlined protocol (compared to the random primed second strand synthesis) and eliminate the need for RNA removal and extended primer annealing steps.

QuantSeq uses total RNA as input, hence no prior poly(A) enrichment or rRNA depletion is required.

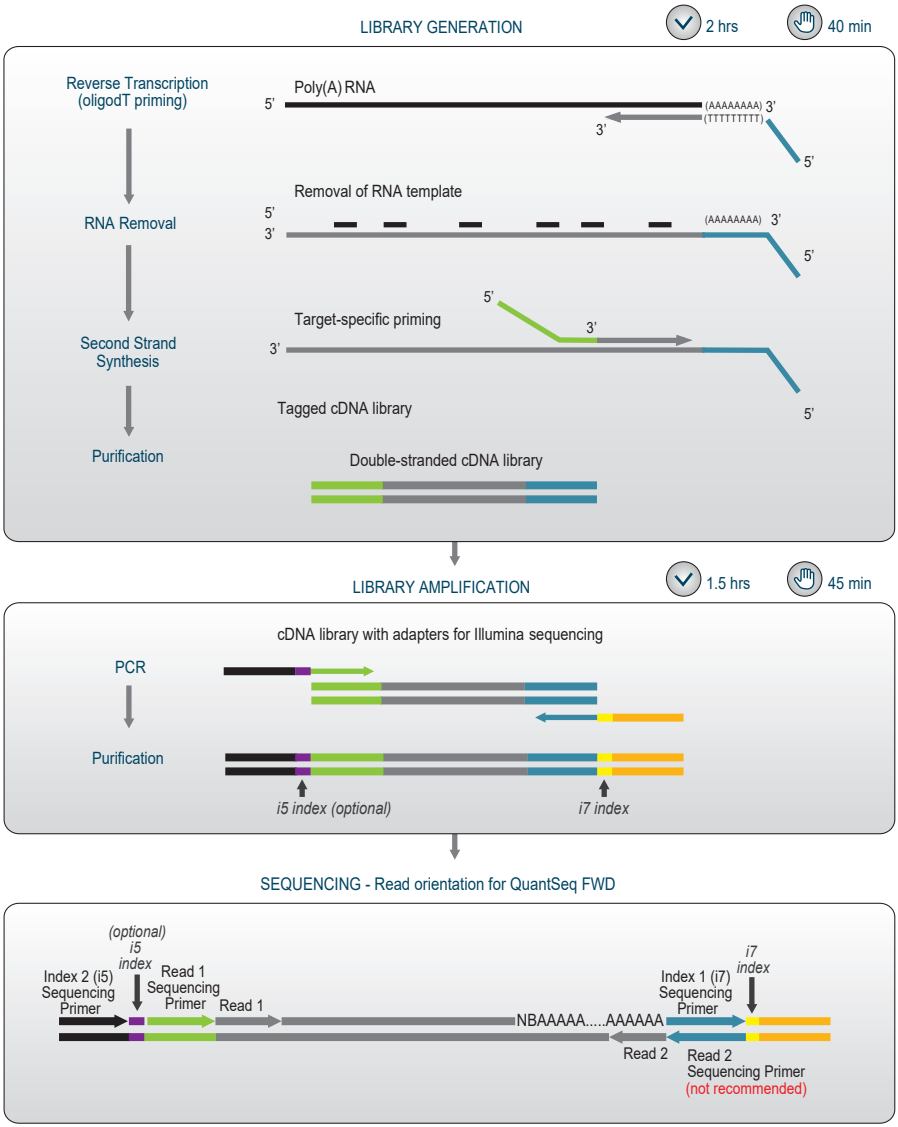

Figure 1. Schematic overview of the QuantSeq-Flex Targeted RNA-Seq library preparation workflow. Read 1 (sequencing starts from the green P5 adapter part) reflects the RNA sequence. The reverse transcription reaction is primed with an oligo(dT) primer and components included in the QuantSeq FWD 3′ mRNA Seq Library prep kits (Cat. No. 015 or 113-115, 129 - 131). QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028) replaces RS ○, SS1 ●, SS2 ●, and E2 ● from the standard QuantSeq FWD Kits and allows for the use of custom primers for second strand snythesis. Library generation and amplfication components from the QuantSeq 3′ mRNA-Seq kits are required for completion of the library prep.

2. Kit Components and Storage Conditions

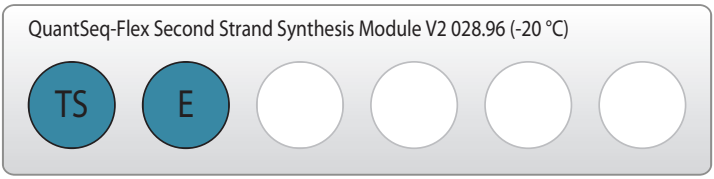

Figure 2. Location of kit components of QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028). TS ● and E ● in this module replace RS ○, SS1 ●, SS2 ●, and E2 ● from QuantSeq FWD kits (Cat. No. 015 or 113 - 115, 129 - 131), allowing to add Custom Targeted SSS Primers.

QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028)	Tube Label	Volume*	Storage
		96 preps	
Target-Specific Second Strand Synthesis Mix	**TS** ●	740 µl	-20 °C
Enzyme Mix	**E** ●	106 µl	-20 °C

*including ≥10 % surplus

NOTE: The **QuantSeq-Flex Second Strand Synthesis Module is not a stand-alone kit**. It is an add-on module for the QuantSeq FWD kits (Cat. No. 015, 113 - 115, 129 - 131) and requires the components therein for functionality.

TS ●, and **E** ●, in the QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028) replace **RS** ○, **SS1** ●, **SS2** ●, and **E2** ● from QuantSeq FWD kits. **Custom Targeted SSS Primers** need to be designed and added by the user.
Lexogen also offers a service for the QuantSeq-Flex custom primer design. Please contact services@lexogen.com.

NOTE: For user-supplied consumables and equipment needs, please refer to the QuantSeq 3′ mRNA-Seq Library Prep Kit for Illumina User Guide.

3. Detailed Protocol

Preparation

Target-specific Second Strand cDNA Synthesis
TS ● – thawed at RT **E** ● – keep on ice or at -20 °C
98 °C, 2 min 42 - 72 °C, 60 sec 72 °C, 5 min; hold at 10 °C

QuantSeq-Flex Targeted Second Strand Synthesis

ATTENTION: QuantSeq FWD-generated first strand cDNA (Cat. No. 015, 113 - 115, 129 - 131) is required as input for targeted second strand synthesis using the Cat. No. 028.

QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028) replaces **RS** ○, **SS1** ●, **SS2** ●, and **E2** ● from the standard QuantSeq FWD kits and allows for the use of custom primers. Target-Specific Second Strand Synthesis Mix (**TS** ●) does not contain primers! 2 µl **Custom Targeted SSS Primers** (designed and provided by the user, see Appendix A, p.10 and Appendix B, p.11) must be added.

NOTE: Targeted primers enable a streamlined protocol. No RNA removal is required prior to second strand synthesis and the primer annealing step is significantly faster than with random primers. Hence when using Cat. No. 028 we recommend placing the Purification Solutions (**PB, EB, PS**) already at room temperature before starting the First Strand cDNA Synthesis (before step 1, User Guide (015UG009 or 113UG227) to give them enough time to equilibrate.

➡	First Strand cDNA Synthesis is carried out according to the standard QuantSeq FWD User Guide (015UG009 or 113UG227) steps 1 to 4 .
5	Prepare a mastermix containing 7 µl of Target-Specific Second Strand Synthesis Mix (**TS** ●), 2 µl **Custom Targeted SSS Primers** (designed and provided by user, see Appendix A, p.10 and Appendix B, p.11), and 1 µl Enzyme Mix (**E** ●). As a starting point we recommend using 2 µl of a 7.5 µM **Custom Targeted SSS Primer** solution.
6	Add 10 µl of this **TS / Custom Targeted SSS Primers / E** mastermix to the First Strand Synthesis reaction from step 4. Mix well.
7	Incubate the reaction for 2 minutes at 98 °C, then allow the primers to anneal for 60 seconds at 45 - 72 °C (depending on the primer Tm) and complete the second strand synthesis by incubating for 5 minutes at 72 °C; shortly hold at 10 °C or store at - 20 °C. ☞ Safe stopping point.

8 Add 20 µl of properly resuspended Purification Beads (**PB**) and 12 µl Purification Solution (**PS**) to each reaction, mix well, and incubate for 5 minutes at room temperature. Continue directly with the purification step 13. **ATTENTION:** Skip step 12 and start directly at step 13. If samples were stored at step 7 equilibrate these to room temperature before restarting the protocol.

Proceed with Purification (step 13) as described in the QuantSeq FWD User Guide (015UG009 or 113UG227).

4. Short Procedure - Targeted Priming V2

ATTENTION: Spin down solutions before opening tubes or plates!

15 min Second Strand Synthesis

Standard Input	
	Second Strand cDNA Synthesis with QuantSeq Flex First Strand Synthesis Module V2
☐	Prepare a mastermix with 7 µl **TS** ●, 2 µl **Custom Targeted Primers**, and 1 µl **E** ● per reaction, mix well.
☐	Add 10 µl **TS** / **Custom Targeted Primers** / **E** premix per 20 µl RT reaction (steps 1 to 4 from QS FWD, User Guide (015UG009 or 113UG227), mix well.
☐	Incubate 2 min at 98 °C; 60 sec at 45 - 72 °C (annealing temperature depends on the custom primers); 5 min at 72 °C; shortly hold at 10 °C or store at -20 °C. ☞ Safe stopping point.
☐	Add 20 µl **PB** and 12 µl **PS** per reaction, mix well, incubate 5 min at RT.
	Proceed to Purification step 13 as described in the QuantSeq FWD User Guide (015UG009 or 113UG227)

5. Appendix A: Primer Design

Target-specific second strand primers can be used with the QuantSeq-Flex Second Strand Synthesis Module. Primers for multiple targets can be combined into a single assay. To ensure off-target effects are minimized we highly recommend checking your designed, targeted primers using the NCBI Primer Blast tool available at https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome. Use the Primer Pair Specificity Check and run your primers against the RefSeq database (not just RefSeq mRNA). Primer specificity stringency settings can be adjusted regarding the allowed mismatches and positions of the mismatch within the primer.

Second Strand Synthesis

Any primer used for first strand cDNA synthesis has to be designed with a partial Illumina P5 adapter extension. Adapter sequences are kept short pre-PCR in order to allow for efficient removal of short fragments during the subsequent purification steps (step 13 and 16). The full Illumina P7 (Read 2) adapter sequence will only be introduced during PCR (step 28) of the QuantSeq FWD 3' Seq Library Prep.

Partial Illumina P5 Adapter Sequence (Read 1) for Second Strand Synthesis Primer:

5' CACGACGCTCTTCCGATCT - (NNNNNN(NN)) - Target sequence (= RNA-sequence) 3'

Here the target sequence has to be the RNA-sequence in question.

The chosen target sequence should be as specific as possible with a Tm that is as close as possible to the intended reaction temperature. The Tm of the targeted primers should be within the range of the potential annealing temperature (45 °C - 72 °C). In most cases 20 nt are enough. Target-specific primer sequences should not exceed a length of 50 nt. The entire primer including the Illumina adapter sequence should not exceed 75 nt. The optimal primer length is 39 - 50 nt (19 nt Illumina-sequence + 20 - 31 nt targeted sequence).

For low quality, degraded, and FFPE RNA samples, take into consideration that your targeted second strand synthesis primers should be located near the 3' end (optimal within 100 - 200 nucleotides upstream of the poly(A) tail).

Optionally, a 6 - 8 nt long molecular index (NNNNNN(NN)) can be introduced between adapter sequence and target sequence. This way, PCR duplication events can be distinguished from unique priming events. Also by using this random sequence, cluster calling can be easily accomplished on Illumina platforms. Illumina platforms rely on the initial rounds of sequencing for cluster calling and that an even nucleotide sequence (25 % of A, C, G, and T) is maintained at each of these positions. If these random nucleotides are not included, be sure to design and combine your targeted primers in such a way that the first 5 nt are equally balanced within the final lane mix.

6. Appendix B: Primer Concentrations

Primer concentrations should be optimized for second strand synthesis in order to maximize library generation efficiency and yield. Multiple transcripts or genes can be targeted simultaneously by preparing a mix of all target-specific oligos to add. Optimizing annealing temperatures for each step is also recommended in order to maximise the specificity of targeted library generation.

The concentration of a target-specific second strand synthesis primer should be 0.5 µM final concentration (i.e., 2 µl of 7.5 µM Custom Targeted Primer). The total concentration of all second strand synthesis primers should not exceed 2 µM. The higher the primer concentration, the higher the likelihood of unspecific binding.

The exact primer concentration and annealing temperature strongly depends on the custom primer(s) used and has to be optimized accordingly. The annealing temperature should be chosen according to the Tm of the targeted primers and can range from 45 - 72 °C. The extension temperature should be 72 °C.

7. Appendix C: Library Quality Control

Quality control of finished QuantSeq libraries is highly recommended and should be carried out prior to pooling and sequencing. Please consult the QuantSeq User Guides (015UG009 or 113UG227).

Typical Results - QuantSeq-Flex Second Strand Synthesis with custom priming

QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028) replaces **RS** ○, **SS1** ●, **SS2** ●, and **E2** ● from the standard QuantSeq FWD Kit (Cat. No. 015, 113 - 115, or 129 - 131) and allows the use of custom primers.
As transcripts may have alternative polyadenylation sites multiple peaks may be seen even for single targets.

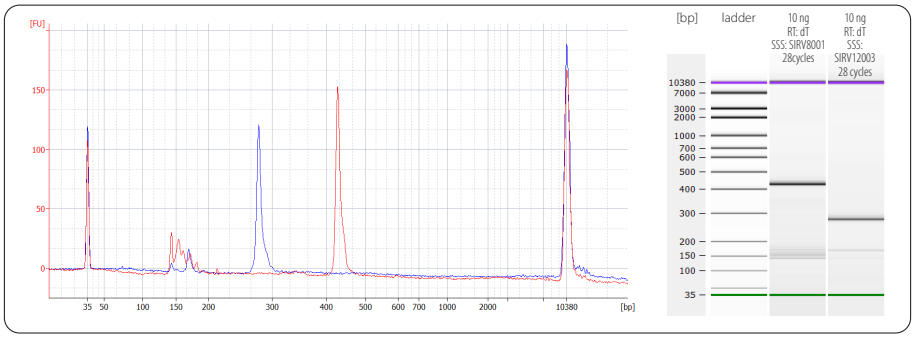

Figure 3. Bioanalyzer traces of QuantSeq FWD oligo(dT)-primed libraries with QuantSeq-Flex Targeted Second Strand Synthesis Module V2 prepared from 10 ng UHRR input RNA containing 0.03% SIRV set 4 spike-in. For targeted second strand synthesis either a SIRV8001 specific primer (red trace) or a SIRV12003 specific primer (blue trace) with an Illumina P5 5' extension was used. EP cycle numbers were determined by qPCR. Libraries were amplified with unique dual indexing (UDIs) and 23 PCR cycles. Illumina Adapter sequences are 144 bp long. In this example primer artifacts are seen below 200 bp.

Overcycling

A second peak in high molecular weight regions (between 1,000 - 9,000 bp) is an indication of overcycling. This could occur if cycle numbers are increased too much to compensate for lower input material. Prevent overcycling by using the qPCR assay as described in the respective Appendix of the QuantSeq User Guides (015UG009 or 113UG227).

8. Appendix D: Data Analysis

In QuantSeq-Flex libraries, Read 1 directly corresponds to the RNA sequence. For more information on the sequences of the libraries consult the QuantSeq User Guides (015UG009 for single indexing and 113UG227 for Unique Dual Indexing).

A basic bioinformatics workflow for the analysis of QuantSeq data is described in the Appendix section of the QuantSeq User Guides (015UG009 or 113UG227).

9. Appendix E: Revision History

Publication No. / Revision Date	Change	Page
028UG350V0100 June 10, 2021	Initial Release.	

LEXOGEN
The RNA Experts

Associated Products:

008 (SPLIT RNA Extraction Kit)
015 (QuantSeq 3′ mRNA-Seq Library Prep Kit for Illumina (FWD))
020 (PCR Add-on Kit for Illumina)
022 (Purification Module with Magnetic Beads)
025, 050, 051, 141 (SIRVs Spike-in RNA Variant Control Mixes)
080 (Reamplification Add-on Kit for Illumina)
081 (UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1))
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)

QuantSeq-Flex Second Strand Synthesis Module V2 · 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

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