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**QuantSeq-Flex Second Strand Synthesis
Module V2**

User Guide

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

028UG350V0102

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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.

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1. Overview

The QuantSeq-Flex Targeted Second Strand Synthesis Module is an Add-on Module that can only be used together with the QuantSeq 3' mRNA-Seq Library Prep Kit FWD.

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 QuantSeq FWD. For more detailed information about this protocol, please refer to the complete QuantSeq FWD User Guide available for download at www.lexogen.com/docs/quantseq. 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 QuantSeq FWD) 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.

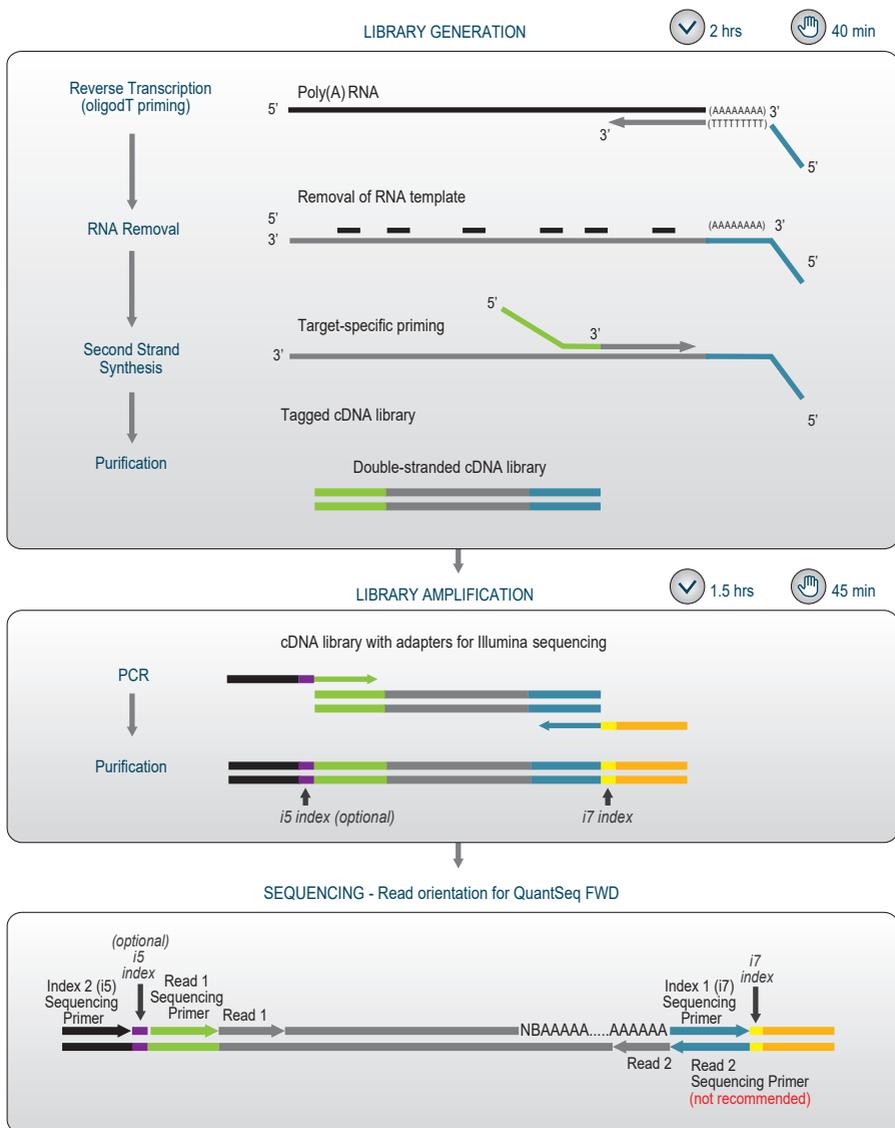


Figure 1. Schematic overview of the QuantSeq-Flex Targeted RNA-Seq library preparation workflow. Read 1 (sequencing starts from the green P5 adapter part) reflects the RNA sequence. The reverse transcription reaction is primed with an oligo(dT) primer and components included in QuantSeq FWD. QuantSeq-Flex Second Strand Synthesis Module V2 replaces RS ○, SS1 ●, SS2 ●, and E2 ● from the QuantSeq FWD Kits and allows for the use of custom primers for second strand synthesis. Library generation and amplification components from QuantSeq FWD are required for completion of the library prep.

2. Kit Components and Storage Conditions

QuantSeq-Flex Second Strand Synthesis Module V2, 028.96 (-20 °C)

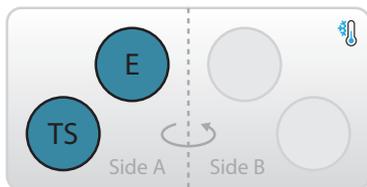


Figure 2. Location of kit components of QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028). TS ● and E ● in this module replace RS ○, SS1 ●, SS2 ●, and E2 ● from QuantSeq FWD kits, allowing to add Custom Targeted SSS Primers.

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 and requires the components therein for functionality.

TS ●, and E ●, in the QuantSeq-Flex Second Strand Synthesis Module V2 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 FWD 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 is required as input for targeted second strand synthesis using QuantSeq-Flex Second Strand Synthesis Module V2.

QuantSeq-Flex Second Strand Synthesis Module V2 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 QuantSeq-Flex Second Strand Synthesis Module V2 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 (191UG444) to give them enough time to equilibrate.



First Strand cDNA Synthesis is carried out according to the QuantSeq FWD User Guide (191UG444) 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 T_m) 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.

Add 20 μ l of properly resuspended Purification Beads (**PB**) and 12 μ l Purification Solution (**PS**) to each reaction, mix well, and incubate for 5 minutes at room temperature.

- 8 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 (191UG444).

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 QuantSeq FWD, User Guide (191UG444), 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 (191UG444)

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 second 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 P5 (Read 1) adapter sequence will only be introduced during PCR (step 28) of the QuantSeq FWD.

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

5' CACGACGCTCTCCGATCT - (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 T_m that is as close as possible to the intended reaction temperature. The T_m 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 T_m of the targeted primers and can range from 45 - 72 $^{\circ}\text{C}$. The extension temperature should be 72 $^{\circ}\text{C}$.

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 FWD User Guide (191UG444).

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

QuantSeq-Flex Second Strand Synthesis Module V2 replaces **RS** ○, **SS1** ●, **SS2** ●, and **E2** ● from the QuantSeq FWD Kit and allows the use of custom primers.

As transcripts may have alternative polyadenylation sites multiple peaks may be seen even for single targets.

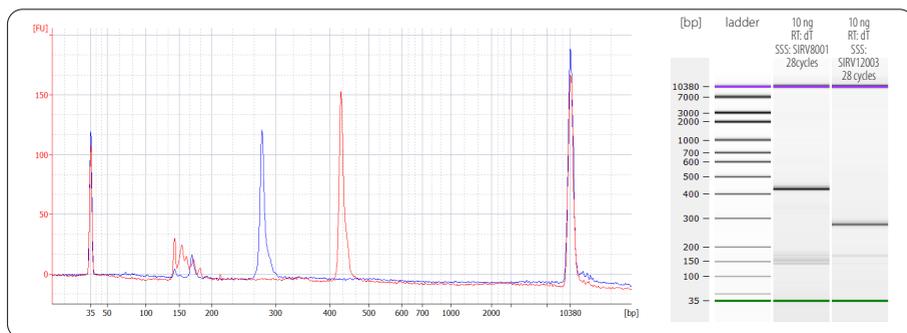


Figure 3. Bioanalyzer traces of QuantSeq FWD oligo(dT)-primed libraries with QuantSeq-Flex Targeted Second Strand Synthesis Module V2 prepared from 10 ng UHRR input RNA containing 0.03 % SIRV set 4 spike-in. For targeted second strand synthesis either a SIRV8001 specific primer (red trace) or a SIRV12003 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 (191UG444).

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 FWD User Guide (191UG444).

A basic bioinformatics workflow for the analysis of QuantSeq data is described in the Appendix section of the QuantSeq FWD User Guide (191UG444).

9. Appendix E: Revision History

Publication No. / Revision Date	Change	Page
028UG350V0102 Jul. 19, 2024	Correction in 5. Appendix A: Primer Design; Second Strand Synthesis; Illumina P5 (Read 1) introduced during PCR.	10
	Updated Cat. No. and User Guide references.	All
028UG350V0101 Jan. 25, 2023	Updated Kit Components Figure 2 and Table to reflect current packaging and storage requirements.	6
028UG350V0100 June 10, 2021	Initial Release.	

Associated Products:

008 (SPLIT RNA Extraction Kit)

022 (Purification Module with Magnetic Beads)

025, 050, 051, 141 (SIRVs Spike-in RNA Variant Control Mixes)

081 (UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1))

191 - 196 (QuantSeq 3' mRNA-Seq V2 Library Prep Kit FWD with UDI)

QuantSeq-Flex Second Strand Synthesis Module V2 · User Guide

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