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Sequencing that counts

QuantSeq-Flex First Strand Synthesis Module V2

User Guide

Catalog Number:
166 (QuantSeq-Flex First Strand Synthesis Module V2 for Illumina)

166UG323V0200

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1. Overview

The QuantSeq-Flex Targeted First Strand Synthesis Module V2 (Cat. No. 166) is an Add-on Module that can only be used together with the QuantSeq 3' mRNA-Seq Library Prep Kit FWD (Cat. No. 015 for single indexing or Cat. No. 113 - 115, 129 - 131 for Unique Dual Indexing).
This User Guide describes the use of the QuantSeq-Flex First Strand Synthesis Module V2 only. To obtain a sequencing-ready RNA-Seq library, this module should be used with the random primed second strand synthesis included in the QuantSeq FWD kits (Cat. No. 015, 113 - 115, 129 - 131).
No special protocol modifications for low input material are required for the denaturation step in the QuantSeq Flex First Strand Synthesis Module V2. High and low input RNA samples can be denatured using the same protocol for added user convenience. When using QuantSeq with oligo(dT) or targeted priming, no prior poly(A) enrichment or rRNA depletion of the total RNA input is required.

The QuantSeq-Flex First Strand Synthesis Module V2 enables:

- Target-specific priming during reverse transcription,
- Increased insert size for standard QuantSeq FWD libraries, when using oligo(dT) priming for reverse transcription, and
- An increased RNA input volume of 13 µl for both oligo(dT) and target-specific priming.

Target-specific priming during reverse transcription
Primers are added separately to the RNA and denatured for efficient hybridization. Reverse transcription can be primed with an Oligo(dT) Primer (included in the QuantSeq-Flex First Strand Synthesis Module V2) or target-specific primers (to be provided by the user). When designing targeted primers, please make sure to include the Illumina P7 sequence (see Appendix A, p.10) at the 5' end of your reverse transcription primers.

Increased insert size for QuantSeq FWD 3' mRNA-Seq libraries
Using the specially formulated First Strand Synthesis Mix with the Oligo(dT) Primer included, the QuantSeq-Flex First Strand Synthesis Module V2 generates 3' mRNA-Seq library fragments with increased insert size, enabling longer read lengths for single-read sequencing. Low sequence complexity of the 3' regions containing common motives such as the polyadenlyation site (PAS) and Upstream Sequence Elements (USE) causes a decrease in uniquely mapping reads, especially when using short read lengths. The longer reads enabled by the QuantSeq-Flex First Strand Synthesis Module V2 compensate for this limitation, increasing the number of usable reads.

Increased input volume for low concentrated RNA samples
By providing a specially formulated First Strand Synthesis Mix, the RNA input volume may be increased to 13 µl, whereas the maximum RNA input volume in the standard QuantSeq 3' mRNA-Seq Library Prep Kit FWD is 5 µl. The QuantSeq Flex First Strand Synthesis Module V2 is therefore highly suited for low concentrated RNA inputs.

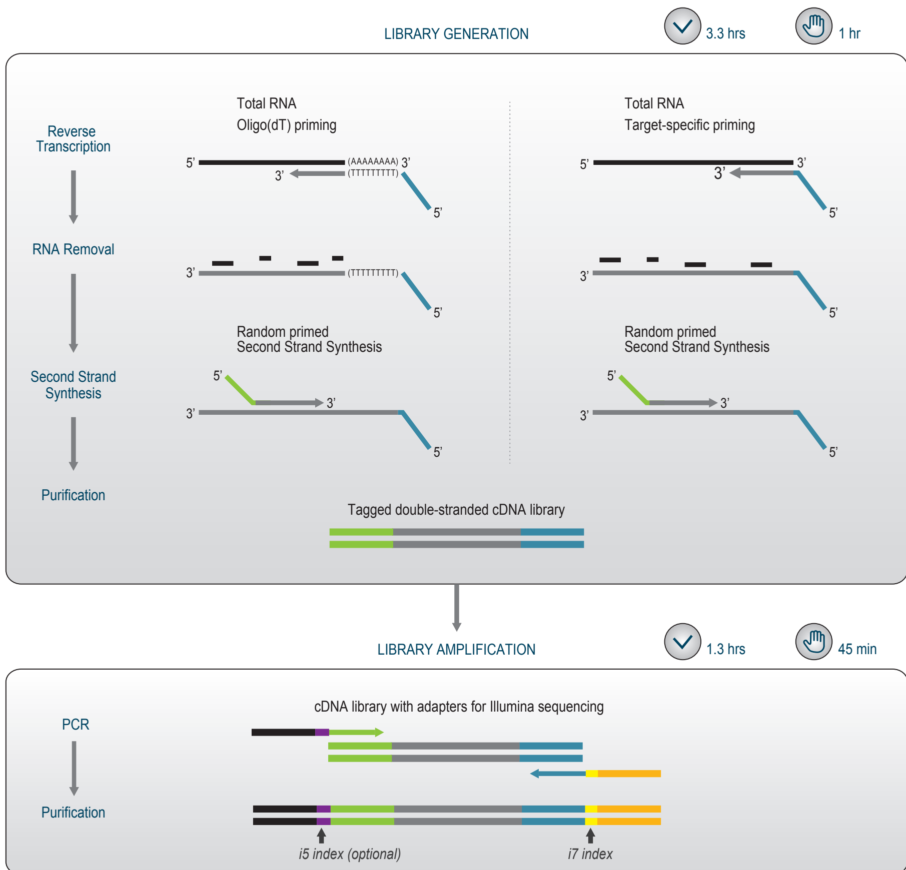

Figure 1. Schematic overview of the QuantSeq-Flex Targeted RNA-Seq library preparation workflow. Read 1 (sequencing starts from the green P5 adapter part) reflects the RNA sequence. The reverse transcription reaction can be primed using either an Oligo(dT) Primer (included in the First Strand Synthesis Module V2, Cat. No. 166) or a target-specific primer (not included, custom designed for desired targets). RT enzyme mix (E1), additional library generation and amplfication components from the QuantSeq 3' mRNA Seq Library prep Kit FWD are required for completion of the Library prep.

For dual-targeted QuantSeq library preparation (targeted reverse transcription and targeted second strand synthesis), please contact Lexogen at support@lexogen.com.

2. Kit Components and Storage Conditions

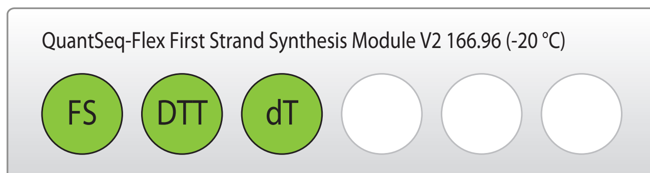

Figure 2. Location of kit components in the QuantSeq-Flex First Strand Synthesis Module V2 (Cat. No. 166). An Oligo(dT) Primer is included for the generation of 3' mRNA-Seq libraries with larger insert sizes and using increased RNA input volumes.

QuantSeq-Flex First Strand Synthesis Module V2 (Cat. No. 166)	Tube Label	Volume*	Storage
		96 preps	
QuantSeq-Flex First Strand cDNA Synthesis Mix	**FS** ●	528.0 µl	-20 °C
DTT	**DTT** ●	52.8 µl	-20 °C
Oligo(dT) Primer	**dT** ●	105.6 µl	-20 °C

*including ≥10 % surplus

NOTE: The **QuantSeq-Flex First Strand Synthesis Module V2 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.

QuantSeq-Flex First Strand cDNA Synthesis Mix (**FS** ●) + DTT (**DTT** ●) + **Oligo(dT) Primer** (**dT** ●), or **FS** ● + **DTT** ●+ **Custom Primers** substitute **FS1** ● and **FS2** ● from the QuantSeq FWD kits. **Custom Primers** need to de designed and provided by the user.

Lexogen also offers a service for the QuantSeq-Flex custom primer design. Please contact services@lexogen.com.

3. Detailed Protocol

Preparation

First Strand cDNA Synthesis
FS ● – thawed at RT **DTT** ● – thawed at RT **dT** ● or Custom Primers – thawed at RT **NOT PROVIDED IN THE MODULE!** QuantSeq FWD core kit is required (Cat. No. 015, or 113 - 115, 129 - 131). **E1** ● – keep on ice or at -20 °C
85 °C, 3 min 42 - 50 °C, 15 min

QuantSeq-Flex First Strand Synthesis - Reverse Transcription

The QuantSeq-Flex First Strand cDNA Synthesis Mix (**FS** ●) does not contain a reverse transcription primer. 1 µl of the provided **Oligo(dT) Primer** (**dT** ●) or 1 µl of **Custom Primers** containing an Illumina-compatible P7 (Read 2) sequence at the 5' end must be added to initiate the reverse transcription. For more information on primer design and concentration see Appendix A, p.10 and Appendix B, p.11 or contact support@lexogen.com.

NOTE: The QuantSeq-Flex First Strand Synthesis Module V2 can also be used if longer inserts or a larger RNA input volume (up to 13 µl) are required for standard QuantSeq FWD libraries. In this case, steps 1 to 4 of the following protocol replace the respective steps in the standard QuantSeq FWD User Guide (015UG009 or 113UG227) using the **Oligo(dT) Primer** (**dT** ●), provided in this kit, instead of **Custom Primers**. Then, continue with RNA removal in step 5 in the standard QuantSeq FWD protocol.

1	Mix 10 ng - 2 µg of total RNA with 1 µl of **Custom Primers** (designed and provided by the user, Appendix A, p.10 and Appendix B, p.11) for targeted reverse transcription libraries, or 1 - 500 ng of total RNA with 1 µl of **Oligo(dT) Primer** (**dT** ●) for QuantSeq FWD 3' mRNA-Seq libraries with increased inset size, in a volume of 13 µl. If necessary, adjust the total volume to 14 µl with RNase-free water. Mix well by pipetting. Ensure the PCR plate or 8-well strip tubes are tightly sealed, and spin down to collect the liquid at the bottom of the wells.
2	Denature the RNA / **Primer** (**dT** ● **or Custom Primers**) mix for 3 minutes at 85 °C in a thermocycler and then cool down to 42 - 50 °C, depending on the primer Tm. Use 42 °C when using **dT** ●. No special protocol modification for low input material is required during denaturation. **ATTENTION:** Leave the samples at the reaction temperature (42 - 50 °C) until step 4 to prevent unspecific primer hybridization.

3. Prepare a mastermix containing 5 µl QuantSeq-Flex First Strand cDNA Synthesis Mix (**FS** ●), 0.5 µl DTT (**DTT** ●), 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 - 50 °C. **ATTENTION:** Do not cool the mastermix on ice!

4. Quickly spin down the denatured RNA / **Primer** 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 - 50 °C and carefully remove the sealing foil / tube caps. Add 6 µl of the **FS / DTT / E1** mastermix to each reaction, mix well, and seal the plate / tubes. If necessary, quickly spin down the liquid at room temperature. Incubate the reactions for 15 minutes at 42 - 50 °C. **ATTENTION:** Proceed immediately to second strand synthesis. Do not cool the samples below room temperature after reverse transcription!

Proceed to RNA removal (step 5) as described in QuantSeq FWD User Guide (015UG009 or 113UG227).

4. Short Procedure - Targeted Priming V2

ATTENTION: Spin down solutions before opening tubes or plates! 30 min Library Generation

Standard Input

First Strand cDNA Synthesis with QuantSeq Flex First Strand Synthesis Module V2

- [ ] Mix 13 µl RNA and 1 µl **Custom Primers** or 1 µl **dT** ●.
- [ ] Incubate for 3 min at 85 °C, then cool to 42 - 50 °C. **Keep samples on thermocycler at 42 °C!**
- [ ] Prepare a mastermix with 5 µl **FS** ●, 0.5 µl **DTT** ●, and 0.5 µl **E1** ● per reaction, mix well and pre-warm at 42 - 50 °C for 2-3 minutes. **Do not cool the mastermix on ice!**
- [ ] Add 6 µl **FS / DTT / E1** mix per reaction, mix well. **Keep samples on thermocycler at 42 - 50 °C when adding mastermix!**
- [ ] Incubate for 15 min at 42 - 50 °C.

Proceed immediately to RNA removal as described in the QuantSeq FWD User Guide (015UG009 or 113UG227)

5. Appendix A: Primer Design

Target-specific primers for reverse transcription (Custom Primers) can be used with the QuantSeq-Flex First 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.

First Strand Synthesis

Any primer used for first strand cDNA synthesis has to be designed with a partial Illumina P7 adapter extension. Adapter sequences are kept short pre-PCR in order to allow for efficient removal of short fragments during the subsequent purification steps (steps 12 - 24). The full Illumina P7 (Read 2) adapter sequence will only be introduced during PCR (step 28) of the QuantSeq FWD 3' mRNA-Seq Library Prep.

Partial Illumina P7 Adapter Sequence (Read 2) for First Strand Synthesis Primer:

5' GTTCAGACGTGTGCTCTTCCGATCT - (NNNNNN(NN)) - Target sequence (= cDNA sequence) 3'

Here the target sequence has to be the reverse complement of the RNA-sequence in question (= cDNA sequence). When using qRT-PCR primers for Flex assay design, the reverse PCR primer should be used for targeted first strand synthesis primer design.

The chosen target sequence should be as specific as possible with a Tm that is as close as possible to the intended reaction temperature (up to 50 °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 45 - 50 nt (20 nt Illumina-sequence + 20 - 25 nt targeted sequence).

Optionally, a 6 - 8 nt long unique molecular index (UMI, $N_{6\text{-}8}$) can be included between the adapter sequence and the target sequence. A paired-end sequencing run is required for read-out of UMIs in this position. With UMIs, PCR duplication events can be distinguished from unique priming events. Note that other sequences of choice (e.g., sample barcodes) can also be placed between the targeted priming and partial adapter sequences.

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 Guides (015UG009 for single indexing and 113UG227 for Unique Dual Indexing).

6. Appendix B: Primer Concentrations

Primer concentrations should be optimized for first 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. 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 first strand synthesis primer should be around 12.5 nM - 1.25 µM final concentration (i.e., 1 µl of a 250 nM - 25 µM target-specific primer). The total concentration of oligos in the first strand synthesis reaction should not exceed 2 µM. The higher the primer concentration, the higher the likelihood of unspecific binding.

The exact primer concentration and reaction temperature (up to 50 °C for first strand synthesis) strongly depends on the custom primer(s) used and has to be optimized accordingly.

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 Guides (015UG009 or 113UG227).

Typical Result for QuantSeq-Flex First Strand Synthesis with Oligo(dT) Priming

The QuantSeq-Flex First Strand Synthesis Module (Cat. No. 166) can be used to increase insert sizes for QuantSeq FWD libraries.

In short: **FS1** ● and **FS2** ● from the standard QuantSeq FWD kit are exchanged with **FS** ●, **DTT** ● and **dT** ● from the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 166). Longer inserts can be generated when RNA is denatured with **dT** ● for 3 minutes at 85 °C and **FS** ● + **DTT** is used for reverse transcription. Longer library sizes may be desired for longer single-read sequencing since increased read-length typically increases unique mapping rates.

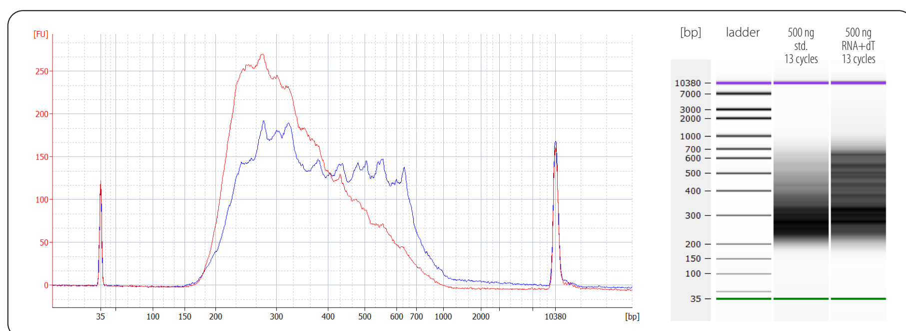

Figure 3. Bioanalyzer traces of QuantSeq FWD (Cat. No. 113) and oligo(dT)-primed QuantSeq-Flex libraries prepared from 500 ng UHRR input RNA. RNA was denatured for 3 minutes at 85 °C, with either 1 µl Oligo(dT) Primer from the QuantSeq-Flex First Strand Synthesis Module V2 (Cat. No. 166; blue trace, RNA+dT), or with 5 µl of the standard QuantSeq FWD FS1 buffer (red trace, std.). Average library size is increased when RNA+dT conditions are used. Libraries were amplified with dual indexing and 13 PCR cycles.

Overcycling

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

8. Appendix D: Data Analysis

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

9. Appendix E: Revision History

Publication No. / Revision Date	Change	Page
166UG323V0200 Jan. 28, 2022	FS buffer change and addition of DTT as separate kit component.	6
	Protocol update: prepare mastermix with 5 µl FS + 0.5 µl DTT + 0.5 µl E1 per rxn (instead of 5.5 µl FS + 0.5 µl E1).	8, 9
166UG323V0100 May 19, 2021	Initial Release.	

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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)
028 (QuantSeq Flex Second Strand Synthesis Module V2)
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)

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