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Enabling complete transcriptome sequencing

QUANT™ SEQ

Sequencing that counts

QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2

User Guide

Catalog Numbers:
026 (QuantSeq-Flex First Strand Synthesis Module for Illumina)
028 (QuantSeq-Flex Second Strand Synthesis Module V2 for Illumina)
033 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2 with First Strand Synthesis Module)
034 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2 with Second Strand Synthesis Module V2)
035 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2 with First and Second Strand Synthesis Modules)
015 (QuantSeq 3′ mRNA-Seq Library Prep Kit for Illumina (FWD))
015 (QuantSeq 3′ mRNA-Seq Library Prep Kit for Illumina (FWD) HT including i5 Dual Indexing Add-on Kit)
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 (SIRVs Spike-in RNA Variant Control Mixes)
047 (i5 Dual Indexing Add-on Kit for QuantSeq/SENSE for Illumina)
081 (UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1))

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ATTENTION: Updated Sequencing Guidelines for Lexogen Libraries

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, QuantSeq-Flex or SENSE 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 Frequently Asked Questions (FAQs).

These recommendations are current as of August 13, 2018. For further information or inquiries please contact info@lexogen.com.

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015UI123V0130

Important recommendations for QuantSeq-Flex Targeted RNA-Seq V2 Library Prep

New changes to the first strand synthesis protocol!

- **The ☞ safe stopping point after reverse transcription, i.e., after step 4 has been removed!** – continue directly to RNA removal or second strand synthesis (for target specific second strand synthesis) after reverse transcription of the libraries. Do not place samples on ice and do not store samples at this point. Cooling the samples below room temperature at this point can cause mishybridization.
- **When storing libraries at -20 °C safe stopping points, ensure the samples are thawed and equilibrated to room temperature before re-starting the protocol.** This is particularly critical before purification steps. Cooler temperatures may negatively affect yields and enhance the presence of unwanted side products.
- **Do not cool the FS2x / E1 mastermix (step 3) but pre-warm for 2 - 3 minutes at 42 °C. Have the RNA / FS1x samples at 42 °C when adding the pre-warmed FS2x / E1 mastermix (step 4). Any drop in temperature can cause mishybridization.** Mix properly. Do not forget to shortly spin down the samples at room temperature before and after adding the FS2x / E1 mastermix. Commence the 42 °C incubation for 15 minutes (or 1 hour for low input RNA (≤10 ng)).

Target-Specific Reverse Transcription (p.13):

1. For low quality, degraded, and FFPE RNA denature the RNA / Custom Targeted Primer mix for only 30 seconds at 85 °C in step 2 .

OligodT Primed Reverse Transcription (p.14):

1. For low quality, degraded, and FFPE RNA prepare a mastermix containing 5 µl FS1x, 5 µl dT, 4.5 µl FS2x, and 0.5 µl E1. Mix well, spin down, and pre-warm at 42 °C on a thermocycler for 2 - 3 minutes.
2. Bring your RNA samples to room temperature while the mastermix is pre-warming.
3. Spin down the pre-warmed FS1x / dT / FS2x / E1 mastermix and add 15 µl to each RNA sample. Mix, seal the plate or strip-tubes, spin down briefly at room temperature, and then commence the 42 °C incubation for 15 minutes (or 1 hour for low input RNA (≤10 ng)).

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LITERATURE CITATION
For any publication using this product, please refer to it as Lexogen's QuantSeq-Flex Targeted RNA-Seq Kit V2.

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1. Overview

Lexogen's QuantSeq FWD kit (Cat. No. 015) is available with flexible modules for First Strand and/or Second Strand cDNA Synthesis, allowing the use of custom primers. This renders the kit suitable for targeted sequencing. The kit provides a library preparation protocol designed to generate Illumina-compatible libraries from total RNA within 4.5 hours. The QuantSeq-Flex protocol generates only one fragment per transcript, resulting in extremely accurate gene expression values, regardless if oligodT priming or target-specific priming is used during first strand synthesis. QuantSeq-Flex Modules are compatible with QuantSeq Forward (FWD, Cat. No. 015) reagents and Read 1 linker sequence is introduced by the second strand synthesis primer, hence NGS reads directly correspond to the mRNA sequence. Primers are added separately to the reverse transcription (RT) as well as to the second strand synthesis reaction (SSS), allowing for maximum flexibility. Reverse transcription can be primed with an oligodT primer (included in the kit) or target-specific primers (to be provided by the user), and uses the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026). When designing targeted primers, please be advised to include the Illumina P7 sequence (see Appendix D, p.28) at the 5′ end of your reverse transcription primers. Second strand synthesis can either be initiated by random priming simply by using the second strand synthesis protocol from QuantSeq FWD or by target-specific primers (to be provided by the user), and uses the QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028). The Illumina P5 sequence (see Appendix D, p.28) has to be included at the 5′ end of the targeted second strand synthesis primers.

With this highly flexible QuantSeq kit the following types of libraries can be generated:

	Random Priming in SSS	Target-Specific Priming in SSS
OligodT Priming in FSS	QuantSeq 3′ mRNA-Seq (FWD)	Targeted 3′ mRNA-Seq
Target-Specific Priming in FSS	Targeted RNA-Seq (enables identification of novel fusions)	Targeted RNA-Seq (known targets detectable only)

FSS: First Strand Synthesis; SSS: Second Strand Synthesis

QuantSeq uses total RNA as input, hence no prior poly(A) enrichment or rRNA depletion is required, unless if using random priming for reverse transcription, where a depletion may be advisable. Information regarding input RNA requirements can be found in Appendix A, p.24.

With the QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028) target-specific second strand synthesis can be performed. Targeted 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.

No purification is required between first and second strand synthesis. Second strand synthesis is followed by a magnetic bead-based purification step. The library is then amplified, introducing the sequences required for cluster generation (see Appendix M, p.41, for a schematic representation of the finished library).

Up to 96 i7 multiplexing indices are included in the QuantSeq kits and are introduced during the PCR amplification step (Appendix L, p.39). Dual indexing, using the i5 Dual Indexing Add-on Kits for QuantSeq/SENSE (5001-5096) (Cat. No. 047) with 96 i5 indices, 96 unique dual-indexed libraries or up to 9,216 different i5 / i7 index combinations are possible (see the respective In-

struction Manual (047IM109)).
Library quality control and quantification can be performed with standard protocols and is further discussed in Appendix G, p.32.
Libraries are compatible with single-read and paired-end sequencing. However, when using oligodT primed first strand synthesis, we do not recommend paired-end sequencing as Read 2 would start with the poly(T) stretch and leading to reduced sequencing quality. When using targeted primers for second strand synthesis please consider the requirements for cluster calling on Illumina platforms (see Appendix D, p.28).
QuantSeq-Flex maintains strand-specificity and allows mapping of reads to their corresponding strand on the genome. The kit includes magnetic beads for the purification steps and hence is compatible with automation.
Data can be analyzed with a number of standard bioinformatics pipelines. Special considerations for the analysis of QuantSeq data, such as read orientation, are presented in Appendix N, p.42.

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 either be primed using an oligodT primer (included in the kit) or a target-specific primer (not included, custom designed for desired targets). Second strand synthesis can either be initiated by random priming (included in the kit) or by using a target-specific primer (not included, custom designed for desired targets). The RNA removal step is only required for random primed second strand synthesis and is omitted when using target-specific primers. Depending on the combination of different priming options four different libraries can be generated.

2. Kit Components and Storage Conditions

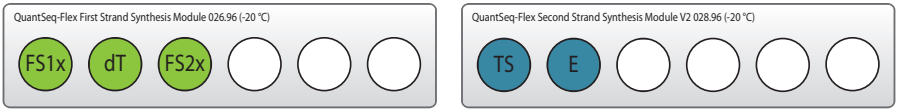

Figure 2. Location of kit components of QuantSeq-Flex First Strand (Cat. No. 026) and Second Strand (Cat. No. 028) Synthesis Modules. QuantSeq-Flex First Strand Synthesis Module can be used to substitute FS1 and FS2 from QuantSeq 015 FWD, allowing to add custom primers. An oligodT primer is included as control. QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028) replaces RS, SS1, SS2, and E2 from the standard QuantSeq FWD Kit and allows to add custom primers.

QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026, 033, 035)	Tube Label	Volume* 96 preps	Storage
QuantSeq-Flex First Strand cDNA Synthesis Mix 1	FS1x	528 µl	-20 °C
OligodT Primer	dT	528 µl	-20 °C
QuantSeq-Flex First Strand cDNA Synthesis Mix 2	FS2x	476 µl	-20 °C

*including 10 % surplus

QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028, 034, 035)	Tube Label	Volume* 96 preps	Storage
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 Modules are not stand-alone kits. They are add-on modules for the QuantSeq 015 FWD kit (kit components are listed on p.7).
The QuantSeq-Flex First Strand Synthesis Module can be used with the second strand synthesis components included in the QuantSeq 015 FWD Kit. Also, the QuantSeq-Flex Second Strand Synthesis Module V2 can be used with the first strand synthesis components included in the QuantSeq 015 FWD Kit, depending on what kind of library is to be generated. The QuantSeq-Flex First Strand Synthesis Module and the QuantSeq-Flex Second Strand Synthesis Module V2 can also be combined for targeted RNA-Seq (target-specific priming during first and second strand cDNA synthesis).

ATTENTION: If using targeted primers for second strand synthesis make sure that at least the first 5 nucleotides of Read 1 meet the balance required for cluster calling (see Appendix D, p.28). Alternatively, include a random sequence of 5 - 8 nt, which may also function as a molecular barcode, between linker sequence and target sequence.

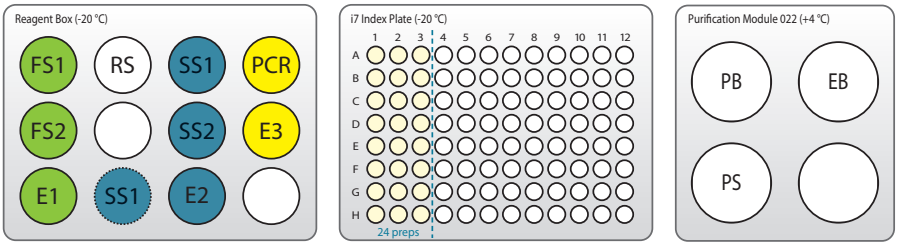

Figure 3. Location of kit components for QuantSeq FWD (Cat. No. 015). For the 24 prep QuantSeq 015 kit the dotted SS1 tube is missing and the i7 Index Plate is only filled with indices 7001-7024 (up to the blue dotted line). All basic kits include Purification Modules. QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026) can be used to substitute FS1 and FS2 from QuantSeq 015 FWD, allowing to add custom primers. The QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028) replaces RS, SS1, SS2, and E2 from the standard QuantSeq FWD Kit and allows to add custom primers.

QuantSeq 015 FWD Reagent Box (Cat. No. 015, 033, 034, 035)	Tube Label	Volume*		Storage
		24 preps	96 preps	
First Strand cDNA Synthesis Mix 1	**FS1** ●	132 µl	528 µl	-20 °C
First Strand cDNA Synthesis Mix 2	**FS2** ●	251 µl	1,004 µl	-20 °C
Enzyme Mix 1	**E1** ●	13.2 µl	53 µl	-20 °C
RNA Removal Solution	**RS** ○	132 µl	528 µl	-20 °C
Second Strand Synthesis Mix 1	**SS1** ●	264 µl	1056 µl	-20 °C
Second Strand Synthesis Mix 2	**SS2** ●	106 µl	423µl	-20 °C
Enzyme Mix 2	**E2** ●	26.4 µl	106 µl	-20 °C
PCR Mix	**PCR** ●	185 µl	740 µl	-20 °C
Enzyme Mix 3	**E3** ●	27 µl	106 µl	-20 °C
i7 Index Plate (96-well plate)	**7001-7096**	5 µl / reaction		-20 °C
Purification Module (Cat. No. 022) included in the kit				
Purification Beads	**PB**	1,320 µl	5,280 µl	+4 °C
Purification Solution	**PS**	2,568 µl	10,349 µl	+4 °C
Elution Buffer	**EB**	2,904 µl	11,616 µl	+4 °C / -20 °C

*including 10 % surplus

Upon receiving the QuantSeq Kit, store the Purification Module (Cat. No. 022), containing **PB**, **PS**, and **EB** at +4 °C and the rest of the kit in a -20 °C freezer. **REMARK:** **EB** can be stored either at +4 °C or -20 °C.

Before use, check the contents of **SS1** ● and **PS**. If a precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

ATTENTION: Spin down the i7 Index Plate before opening! Visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired indices. Avoid cross contamination! Reseal opened wells after usage to prevent cross contamination! Each well of the i7 Index Plate is intended for single-use only!

3. User-Supplied Consumables and Equipment

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

Reagents / Solutions

- 80 % fresh ethanol (washing of Purification Beads, **PB**).
- Optional: SYBR Green I (Sigma-Aldrich, Cat. No. S9430), 10,000x in DMSO for qPCR.
- Optional: PCR Add-on Kit for Illumina (Cat. No. 020) for qPCR.

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 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 (Agilent Technologies 2100 Bioanalyzer).
- qPCR machine and library standards (for endpoint determination and 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 A, p.24 for more information on RNA quality.
Consult Appendix H, p.33 for information on library quantification methods.

4. Guidelines

RNA Handling

- RNases are ubiquitous, and special care should be taken throughout the procedure to avoid RNase contamination.
- Use commercial ribonuclease inhibitors (i.e., RNasin, Promega Corp.) to maintain RNA integrity when storing samples.
- Use a sterile and RNase-free workstation or laminar flow hood if available. Please note that RNases may still be present on sterile surfaces and that autoclaving does not completely eliminate RNase contamination. Well before starting a library preparation, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) as per the manufacturer's instructions. **ATTENTION:** Do not forget to rinse off any RNAZap residue with RNAse-free water after usage! Residues of RNAZap may damage the RNA.
- Protect all reagents and your RNA samples from RNases on your skin by wearing a clean lab coat and fresh gloves. Change gloves after making contact with equipment or surfaces outside of the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

Bead Handling

- Beads are stored at +4 °C and must be resuspended before usage. Beads can be resuspended by pipetting up and down several times or by vortexing. When properly resuspended, the solution should have a uniform brown color with no visible clumping on the walls or bottom of the tube or storage bottle.
- Beads may stick to certain pipette tips, in which case removing the beads from the inside of the tip may be impossible. Avoid resuspending by repeated pipetting and instead resuspend by vortexing if this occurs with your tips.
- Beads are superparamagnetic and are collected by placing the plate / tube in a magnetic plate or stand. The time required for complete separation will vary depending on the strength of your magnets, wall thickness of the wells / tubes, viscosity of the solution, and the proximity of the well / tube to the magnet. Separation time may need to be adjusted accordingly. When fully separated, the supernatant should be completely clear and the beads collected at one point or as a ring along the wall of the well / tube, depending on the magnet that was used.
- To remove the supernatant, the plate / tube containing the beads has to stay in close contact with the magnet. Do not remove the plate / tube from the magnetic plate / stand when

removing the supernatant, as the absence of the magnet will cause the beads to go into suspension again.

- When using a multichannel pipette to remove the supernatant, make sure not to disturb the beads. If beads were disturbed, ensure that no beads are stuck to the pipette tip opening and leave the multichannel pipette in the well for an extra 30 seconds before removing the supernatant. This way all beads can be recollected at the magnet and the clear supernatant can be removed.
- In general, beads should not be centrifuged during the protocol. However, should drops of fluid stay on the wall of the reaction tube / plate (e.g., after mixing by vortexing), centrifugation at 2,000 x g for 30 seconds should be carried out before placing the tube / plate on the magnetic stand / plate.
- Allowing the beads to dry out can damage them. Always keep the beads in suspension except for the short period after withdrawing the supernatant, and before adding the next reagent. Beads can be resuspended by vortexing, but make sure that beads are not deposited on the well / tube walls above the level of the liquid, where they can dry during incubation. If necessary, stuck beads can be collected by centrifuging the plate / tube briefly with a suitable benchtop centrifuge.

General

- Unless explicitly mentioned, all steps including centrifugation, should be carried out at room temperature (RT) between 20 °C and 25 °C. Results may be negatively impacted if the protocol is performed at temperatures outside of this range. While reaction set-up is often performed at RT, incubation temperatures are explicitly defined, and must be strictly adhered to.
- To further increase reproducibility and to avoid cross contamination a centrifugation step should be performed after incubations at elevated temperatures and before removing the sealing foil from PCR plates, e.g., step 2.
- Ensure that adequate volumes of all reagents and the necessary equipment are available before beginning the protocol.
- Perform all pipetting steps with calibrated pipettes and always use fresh tips. Pipette carefully to avoid foaming as some solutions contain detergents.
- Thaw all necessary buffers at room temperature or as indicated in the preparation tables at the beginning of each step of the detailed protocol. Mix reagents well by vortexing or pipetting repeatedly and centrifuge briefly with a benchtop centrifuge to collect contents before use.
- Keep Enzyme Mixes at -20 °C until right up before use or store in a -20 °C benchtop cooler.
- Steps requiring a thermocycler have been tested with a maximum ramp speed of 5 °C/sec before denaturation and extension and 2.5 °C/sec during primer annealing. While these ramp speeds are typical for most modern thermocyclers, some models can exceed these rates, and ramp speed may need to be decreased to ensure efficient annealing. Ramp

speeds may be reduced even further in some steps of the protocol to ensure better hybridization.

- When mixing by pipetting, set the pipette to a larger volume. For example after adding 5 µl for random primed second strand synthesis in steps 5 and 10 use a pipette set to 15 µl or 30 µl, respectively, to ensure proper mixing.

Pipetting and Handling of (Viscous) Solutions

- Enzyme Mixes, **SS1** ●, **PB**, and **PS** are viscous solutions which require care to pipette accurately. Mix properly and quickly spin down the tubes to collect all liquid at the bottom of the tube before drawing an aliquot. Be sure to pipette slowly and check the graduation marks on your pipette tips when removing an aliquot.
- When drawing up liquid, the tip should be dipped 3 to 5 mm below the surface of the liquid, always at a 90 degree angle. Do not dip the tip in any further as viscous solutions tend to stick to the outside of the pipette tip.
- Any residual liquid adhering to the tip should be removed by sliding the tip up the wall or edge of the tube from which the liquid was taken. Spin down the tube afterwards again to ensure that all liquid is collected at the bottom of the tube for further storage.
- When dispensing, the pipette should be held at a 45 degree angle, and the tip placed against the side of the receiving vessel.
- When pipetting liquids from bottles, take special care that only the sterile pipette tip touches the bottle opening to prevent introducing RNases or other contaminants. Tips are sterile, whereas the pipette itself is not. If necessary, tilt the bottle to bring the liquid closer to the opening and facilitate pipetting.

Preparation of Mastermixes and Pipetting with Multi-Channel Pipettes

In steps 3, 5 for targeted, and 9 for random primed second strand synthesis, and 25 of the QuantSeq protocol, mastermixes of enzymes and reaction buffers should be prepared. When preparing mastermixes and when using multi-channel pipettes always include a 10 % surplus per reaction in order to have enough solution available for all reactions.
EXAMPLE: Step 3 for 24 preps: use 118.8 µl **FS2x** ● (= 4.5 µl x 24 rxn x 1.1)
+ 13.2 µl **E1** ● (= 0.5 µl x 24 rxn x 1.1)
resulting in a total of 132 µl, which is sufficient for multi-channel pipetting.
All reagents of the QuantSeq kit include a 10 % surplus.

Automation

QuantSeq is compatible with automation and Lexogen provides automated protocols and software for diverse platforms. If you are interested in an automated protocol or need help automating QuantSeq on your NGS workstation, please contact Lexogen (info@lexogen.com).

5. Detailed Protocol

5.1 Library Generation

Preparation

First Strand cDNA Synthesis	RNA Removal	Second Strand Synthesis	Purification
FS1x ● – thawed at RT **dT** ● – thawed at RT **FS2x** ● – thawed at RT **FS1** ● – thawed at RT **FS2** ● – thawed at RT **E1** ● – keep on ice or at -20 °C	**RS** ○ – thawed at RT **SKIP IF using target-specific second strand synthesis!**	**Target-specific priming:** **TS** ● – thawed at RT **E** ● – keep on ice or at -20 °C **Random priming:** **SS1** ● – thawed at 37 °C **SS2** ● – thawed at RT **E2** ● – keep on ice or at -20 °C	**PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – stored at +4 °C
85 °C, 3 min 42 - 50 °C, 15 min	95 °C, 10 min cool down to 25 °C	**Target-specific priming:** 98 °C, 2 min 42 - 72 °C, 60 sec 72 °C, 5 min; hold at 10 °C **Random priming:** 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

First strand cDNA synthesis can be either initiated by target-specific priming (primers designed and provided by user, see also Appendix D, p.28 and Appendix E, p.30) or by oligodT priming.

NOTE: Protocol modifications are recommended for low quality, degraded, and FFPE RNA samples. These are indicated as **"REMARK"** in the respective protocol steps (see also Appendix C, p.27).

Target-Specific Reverse Transcription (QuantSeq-Flex First Strand Synthesis Module Cat. No. 026)

ATTENTION: For targeted priming during the reverse transcription substitute **FS1** ● and **FS2** ● from the QuantSeq FWD reagent box (Cat. No. 015) with **FS1x** ● and **FS2x** ● from the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026). **FS1x** ● does not contain a reverse transcription primer, so any primer containing an Illumina-compatible P7 (Read 2) sequence at its 5' end can be added with a volume of up to 5 µl. For primer design and concentration see Appendix D, p.28 and Appendix E, p.30.

NOTE: For target-specific second strand synthesis using the QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028) place the Purification Solutions (**PB**, **EB**, **PS**) at room temperature before starting the first strand cDNA synthesis to give them enough time to equilibrate.

1. Mix 10 ng - 2 µg of total RNA in a volume of 5 µl, with 5 µl **Custom Targeted Primers** (designed and provided by user, see Appendix D, p.28 and Appendix E, p.30) in a PCR plate or 8-well strip tubes. If necessary, adjust the total volume to 10 µl with RNase-free water. Mix well by pipetting. Ensure the plate or PCR strips / tubes are tightly sealed, and spin down to collect the liquid at the bottom of the wells.

2. Denature the RNA / **Custom Targeted Primer** mix for 3 minutes at 85 °C in a thermocycler and then cool down to 42 - 50 °C, depending on the primer Tm. **ATTENTION:** Leave the samples at the reaction temperature (42 - 50 °C) until step 4 to prevent unspecific primer hybridization. **REMARK:** For low quality, degraded, and FFPE RNA denature the RNA / **Custom Targeted Primer** mix for only 30 seconds at 85 °C.

3. Prepare a mastermix containing 5 µl QuantSeq-Flex First Strand cDNA Synthesis Mix 1 (**FS1x** ●), 4.5 µl QuantSeq-Flex First Strand cDNA Synthesis Mix 2 (**FS2x** ●), 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 / **Custom Targeted 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 10 µl of the **FS1x / FS2x / 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. **OPTIONAL:** For low quality, degraded, and FFPE RNA, extend the incubation time to 1 hour at 42 - 50 °C. **ATTENTION:** Proceed immediately with targeted (p.15) or random primed second strand synthesis (p.16). Do not cool the samples below room temperature after reverse transcription!

OligodT Primed Reverse Transcription (QuantSeq-Flex First Strand Synthesis Module Cat. No. 026)

NOTE: The QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026) also contains a separate OligodT Primer (**dT** ●) containing an Illumina-compatible P7 (Read 2) linker sequence at its 5'end for control experiments. The QuantSeq-Flex First Strand Synthesis Module with oligodT priming in combination with the random primed second strand synthesis from the standard QuantSeq FWD Kit (Cat. No. 015) can be used to increase the insert size of QuantSeq 3' mRNA-Seq libraries due to the different reagent volumes (see Figure 5 in Appendix I, p.34).

NOTE: For target-specific second strand synthesis using the QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028), we recommend placing the Purification Module (**PB**, **PS**, **EB**) already at room temperature before starting the First Strand cDNA synthesis to give them enough time to equilibrate.

1. Mix 10 ng - 2 µg of total RNA in a volume of 5 µl, with 5 µl QuantSeq-Flex First Strand cDNA Synthesis Mix 1 (**FS1x** ●) and 5 µl OligodT Primer (**dT** ●) in a PCR plate or 8-well strip tubes. If necessary, adjust the total volume to 10 µl with RNase-free water. Mix well by pipetting. Ensure the plate or PCR strips / tubes are tightly sealed, and spin down to collect the liquid at the bottom of the wells. **REMARK:** For low quality, degraded, or FFPE RNA, do not add **FS1x** ● and **dT** ● to the RNA. Instead place the RNA samples at room temperature while preparing the mastermix (see below).

2. Denature the RNA / **FS1x** / **dT** 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 quality, degraded and FFPE RNA (see Appendix C, p.27).

3. Prepare a mastermix containing 4.5 µl QuantSeq-Flex First Strand cDNA Synthesis Mix 2 (**FS2x** ●) 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 (**FS1x** ●), 5 µl OligodT Primer (**dT** ●), 4.5 µl **FS2x** ●, and 0.5 µl **E1** ● per sample. Mix well, spin down, and pre-warm for 2 - 3 minutes at 42 °C. **ATTENTION:** Do not cool mastermixes on ice!

4. Quickly spin down the denatured RNA / **FS1** samples from step 2 at room temperature to make sure all liquid is collected at the bottom of the wells. Place the samples back onto the thermocycler at 42 °C and carefully remove the sealing foil / tube caps. Add 5 µl of the **FS2x / E1** mastermix to each reaction, mix by pipetting, and seal the plate / tubes. If necessary, quickly spin down the liquid at room temperature. Incubate the reactions for 15 minutes at 42 °C. **REMARK:** If step 2 is skipped, add 15 µl of the pre-warmed **FS1x** / **dT** / **FS2x** / **E1** mastermix to each 5 µl RNA sample. Seal the plate / tubes and mix with gentle vortexing. Quickly spin down at room temperature and incubate the reactions for 15 minutes at 42 °C. **OPTIONAL:** For low quality, degraded, and FFPE RNA, extend the incubation time to 1 hour at 42 °C. **ATTENTION:** Proceed immediately with targeted (p.15) or random primed second strand synthesis (p.16). Do not cool the samples below room temperature after reverse transcription!

OligodT Primed Reverse Transcription (QuantSeq FWD Kit Components Cat. No. 015)

An oligodT primer containing an Illumina-compatible P7 (Read 2) linker sequence at its 5' end is already included in **FS1** ●. After hybridization to the RNA, reverse transcription is performed.

NOTE: For target-specific second strand synthesis using the QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028) we recommend placing the Purification Module (**PB**, **PS**, **EB**) already at room temperature before starting the First Strand cDNA synthesis to give them enough time to equilibrate.

1	Mix 10 ng - 2 µg of total RNA in a volume of 5 µl, with 5 µl First Strand cDNA Synthesis Mix 1 (**FS1** ●) in a PCR plate or 8-well strip tubes. If necessary, adjust the total volume to 10 µl with RNase-free water. Mix well by pipetting. Ensure the plate or PCR strips / tubes are tightly sealed, and spin down to collect the liquid at the bottom of the wells. **REMARK:** For low quality, degraded, or FFPE RNA, do not add **FS1** ● to the RNA. Instead place the RNA samples at room temperature while preparing the mastermix (see below).
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 quality, degraded, or FFPE RNA.
3	Prepare a mastermix containing 9.5 µl First Strand cDNA Synthesis Mix 2 (**FS2** ●) and 0.5 µl Enzyme Mix 1 (**E1** ●) per reaction. Mix well, spin down, and pre-warm the mastermix for 2 - 3 minutes at 42 °C. **REMARK:** If step 2 is skipped, prepare a mastermix containing 5 µl First Strand cDNA Synthesis Mix 1 (**FS1** ●), 9.5 µl **FS2** ●, and 0.5 µl **E1** ● per sample. Mix well, spin down, and pre-warm for 2 - 3 minutes at 42 °C. **ATTENTION:** Do not cool mastermixes on ice!
4	Quickly spin down the denatured RNA / **FS1** samples from step 2 at room temperature to make sure all liquid is collected at the bottom of the wells. Place the samples back onto the thermocycler at 42 °C and carefully remove the sealing foil / tube caps. Add 10 µl of the **FS2** / **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 °C. **REMARK:** If step 2 is skipped, add 15 µl of the pre-warmed **FS1** / **FS2** / **E1** mastermix to each 5 µl RNA sample. Seal the plate / tubes and mix with gentle vortexing. Quickly spin down at room temperature and incubate the reactions for 15 minutes at 42 °C. **OPTIONAL:** For low quality, degraded, or FFPE RNA, extend the incubation time to 1 hour at 42 °C. **ATTENTION:** Proceed immediately to the RNA removal step! Do not cool the samples below room temperature after reverse transcription!

Second Strand Synthesis

During this step the library is converted to dsDNA. Second strand synthesis can either be initiated with target-specific primers (to be provided by the user) and the QuantSeq-Flex Second Strand Synthesis Module V2 or with random priming simply using the QuantSeq FWD Kit (Cat. No 015).

Target-Specific Second Strand Synthesis (No RNA Removal Required) (QuantSeq-Flex Second Strand Synthesis Module V2 Cat. No. 028)

ATTENTION: 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) and allows for the use of custom primers. For primer design and concentration guidelines see Appendix D, p.28 and Appendix E, p.30.

ATTENTION: 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 the random primers.

NOTE: With this protocol version we recommend placing the Purification Solutions (**PB, EB, PS**) already at room temperature before starting the First Strand cDNA Synthesis (before step 1) to give them enough time to equilibrate.

5	Prepare a mastermix containing 7 µl of Target-Specific Second Strand Synthesis Mix (**TS** ●), 2 µl **Custom Targeted Primers** (designed and provided by user, see Appendix D, p.28 and Appendix E, p.30), and 1 µl Enzyme Mix (**E** ●). As a starting point we recommend using 2 µl of a 7.5 µM **Custom Targeted Primer** solution.
6	Add 10 µl of this **TS / Custom Targeted 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 steps 13 to 24 as described on p.18. **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.

Random Primed Second Strand Synthesis (RNA Removal Required) (QuantSeq FWD Kit Components Cat. No. 015)

ATTENTION: This protocol is identical to the QuantSeq FWD (Cat. No. 015) Second Strand Synthesis protocol steps 5 - 12 . Before random primed second strand synthesis, the RNA has to be removed.

RNA Removal

During this step the RNA template is degraded, which is essential for efficient second strand synthesis. After the first strand synthesis reaction, quickly spin down the plate to make sure all liquid is collected at the bottom of the wells before removing the sealing foil.

5	Add 5 µl RNA Removal Solution 1 (**RS** ○) directly to the first strand cDNA synthesis reaction. Mix well and reseal the plate using a fresh foil. **REMARK:** Use a pipette set to 15 µl for efficient mixing.
6	Incubate 10 minutes at 95 °C, then cool down to 25 °C. Spin down the plate at room temperature and carefully remove the sealing foil.

Random Primed Second Strand Synthesis

Second strand synthesis is initiated by a random primer containing an Illumina-compatible P5 (Read 1) linker sequence at its 5' end. **ATTENTION:** Second Strand Synthesis Mix 1 (**SS1** ●) is a viscous solution and needs to be mixed thoroughly before use. Thaw at 37 °C. If a precipitate is visible, incubate at 37 °C and mix until buffer components dissolve completely.

NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, **EB**) for step 12 at room temperature to give it enough time to equilibrate.

OPTIONAL: At step 7 the UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1, Cat. No. 081) may be used to include Unique Molecular Identifiers (UMIs) in QuantSeq FWD libraries. The UMI Second Strand Synthesis Mix (**USS** ●) replaces the Second Strand Synthesis Mix 1 (**SS1** ●) from the standard QuantSeq FWD Kit (Cat. No. 015) (see Appendix K, p.37).

7	Add 10 µl Second Strand Synthesis Mix 1 (**SS1** ●). Mix well by pipetting, and seal the plate. **REMARK:** Use a pipette set to 30 µl for efficient mixing.
8	Incubate the plate for 1 minute at 98 °C in a thermocycler, and slowly cool down to 25 °C by setting the ramp speed to 10 % (0.5 °C/second). Incubate the reaction for 30 minutes at 25 °C. Quickly spin down the plate at room temperature 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.

10 Add 5 µl of the **SS2** / **E2** mastermix per reaction. Mix well. **REMARK:** Use a pipette set to 30 µl for efficient mixing.

11 Incubate the reaction at 25 °C for 15 minutes. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The double-stranded library is purified by using magnetic beads to remove all reaction components. The Purification Module (**PB**, **PS**, **EB**) should equilibrate for 30 minutes at room temperature before use. **PB** may have settled and must be properly resuspended before adding them to the reaction.

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

12 Add 16 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature. **NOTE:** Skip this step if target-specific second strand synthesis was carried out (where **PB** and **PS** were added in step 8 p.15).

13 Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear (depends on the strength of your magnet).

14 Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. 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 properly 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. **ATTENTION:** For low input RNA, FFPE, or degraded RNA add only 48 µl **PS** (see Appendix B, p.26 and Appendix C, p.27).

17 Place the plate onto a magnetic plate 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 PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

19 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.

20 Repeat this washing step once for a total of two washes. Make sure to 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 properly in **EB**. Incubate for 2 minutes at room temperature.
23	Place the plate onto a magnetic plate 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. Make sure not to transfer any beads. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point. See Appendix F, p.31 for qPCR options.

5.2 Library Amplification - Single Indexing (i7 only)

Preparation

PCR		Purification	
PCR ●	– thawed at RT	**PB**	– stored at +4 °C
E3 ●	– keep on ice or at -20 °C	**PS**	– stored at +4 °C
i7 Index Plate	– thawed at RT; in 96-well plate; **spin down before opening!**	**80 % EtOH**	– provided by user; **prepare fresh!**
		EB	– stored at +4 °C
Thermocycler	98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec } X best determined by qPCR see Appendix F, p.31 72 °C, 1 min 10 °C, ∞	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, to add unique indices for multiplexing, and to generate sufficient material for quality control and sequencing.

ATTENTION: Cycle numbers may differ depending on the RNA used. At this point, **we strongly recommend performing a qPCR assay to determine the optimal PCR cycle number** for your particular RNA sample type, using the PCR Add-on Kit for Illumina (Cat. No. 020). For details see Appendix F, p.31.

NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, **EB**) for step 29 at room temperature to give them enough time to equilibrate.

OPTIONAL: For dual indexing please follow the protocol for Endpoint PCR as indicated in the i5 Dual Indexing Add-on Kits Instruction Manual (047IM109).

25	Prepare a mastermix containing 7 µl of PCR Mix (**PCR** ●) and 1 µl Enzyme Mix 3 (**E3** ●) per reaction.
26	Add 8 µl of this **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 to make sure all liquid is collected at the bottom of the well. **ATTENTION:** Spin down the i7 Index Plate before opening! Visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired indices. Avoid cross contamination! Reseal opened wells of the i7 Index Plate after usage to prevent cross contamination! **NOTE:** Each well of the i7 Index Plate is intended for single use only!
28	Conduct x cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, x cycles (best determined by qPCR) of: 98 °C for 10 seconds, 65 °C for 20 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, hold at 10 °C. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

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

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

29	Add 30 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature. **ATTENTION:** For low input, degraded, and FFPE RNA add only 27 µl Purification Beads (**PB**) (see Appendix C, p.27).
30	Place the plate onto a magnetic plate, 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 magnetic plate. Make sure that accumulated beads are not disturbed.
32	Add 30 µl of Elution Buffer (**EB**), remove the plate from the magnet and resuspend the beads properly 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 magnetic plate 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 PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

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. Make sure to 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 properly in **EB**. Incubate for 2 minutes at room temperature.
40	Place the plate onto a magnetic plate 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. Make sure not to transfer any beads.
42	At this point, the libraries are finished and ready for quality control (Appendix H, p.33), pooling (for multiplexing see Appendix L, p.39), and cluster generation. For a schematic representation of the library see Appendix M, p.41. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

6. Short Procedure - Targeted Priming V2

ATTENTION: Spin down solutions before opening tubes or plates!

155 min	Library Generation
☐ Mix 5 µl RNA and 5 µl **Custom Targeted Primers**.	Targeted First Strand cDNA Synthesis
☐ Incubate for 3 min at 85 °C, cool to 42 - 50 °C. Use only 30 sec at 85 °C for low quality, degraded, and FFPE RNA samples. **ATTENTION:** Keep samples on thermocycler at 42 - 50 °C.	
☐ Prepare a mastermix with 5 µl **FS1x** ●, 4.5 µl **FS2x** ●, and 0.5 µl **E1** ● per reaction, mix well. **ATTENTION:** Do not cool the mastermix on ice!	
☐ Add 10 µl **FS1x** / **FS2x / E1** mix for targeted priming per reaction, mix well.	
☐ Incubate for 15 min at 42 °C (or up to 50 °C for targeted primers with a high Tm). **OPTIONAL:** May be increased to 1 h for low quality, degraded, or FFPE RNA.	
☐ Prepare a mastermix with 7 µl **TS** ●, 2 µl **Custom Targeted Primers**, and 1 µl **E** ● per reaction, mix well.	Targeted Second Strand cDNA Synthesis
☐ Add 10 µl **TS** / **Custom Targeted Primers** / **E** premix per RT reaction, 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.	Purification (after Targeted Second Strand cDNA Synthesis)
☐ Place on magnet for 2 - 5 min, remove and discard supernatant.	
☐ Add 40 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.	
☐ Add 56 µl **PS** (or 48 µl **PS** for low quality, degraded, and FFPE RNA), mix well, incubate 5 min at RT.	
☐ Place on magnet for 2 - 5 min, remove and discard supernatant.	
☐☐ Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.	
☐ Dry beads for 5 - 10 min. **ATTENTION:** Do not let the beads dry too long!	
☐ Add 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.	
☐ Place on magnet for 2 - 5 min, transfer 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.	

ATTENTION: If using the QuantSeq 015 first strand synthesis components (oligodT primer in FS1) or the QuantSeq 015 second strand synthesis components for random primed second strand synthesis use the Reference Card from the basic kit for the respective sections. Do not forget the RNA removal step before random primed second strand synthesis! **ATTENTION:** Purification differs post targeted and random primed second strand synthesis!

70 min Library Amplification

- ☐ Prepare a mastermix with 7 µl **PCR** ● and 1 µl **E3** ● per reaction, mix well.
- ☐ Add 8 µl **PCR** / **E3** premix to 17 µl of each purified library.
- ☐ Add 5 µl i7 primer (**7001-7096**, from the 96-well plate) for each reaction, mix well.
- ☐ PCR: 98 °C, 30 sec

  98 °C, 10 sec
  65 °C, 20 sec
  72 °C, 30 sec
  } X determine by qPCR; see Appendix F, p.31

  72 °C, 1 min
  10 °C, ∞. ☞ Safe stopping point.

PCR

- ☐ Add 30 µl **PB** (or 27 µl **PB** for low input, FFPE, or low quality RNA) per reaction, mix well, incubate 5 min at RT.
- ☐ Place on magnet for 2 - 5 min, remove and discard supernatant.
- ☐ Add 30 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.
- ☐ Add 30 µl **PS**, mix well, incubate 5 min at RT.
- ☐ Place on magnet for 2 - 5 min, remove and discard supernatant.
- ☐☐ Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.
- ☐ Air dry beads for 5 - 10 minutes. **ATTENTION:** Do not let the beads dry too long!
- ☐ Add 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.
- ☐ Place on magnet for 2 - 5 min, transfer 15 - 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.

Purification

7. Appendix A: RNA Requirements

Potential Contaminants

RNA samples should be free of salts, metal ions, and organic solvents, which can be carried over from RNA extraction. Several sources of contamination can be detected with a UV-Vis spectrophotometer. An acceptably pure RNA sample should have an A260/A280 ratio between 1.8 and 2.1. The A260/A230 ratio should be approximately 2. Several common contaminants including proteins, chaotropic salts, and phenol absorb strongly between 220 and 230 nm and can often be identified as peaks in this region. Contamination with any of these generates a lower A260/230 ratio. Phenol has an additional absorption maximum between 250 and 280 nm, which overlaps that of nucleic acid, so high 230 nm absorbance combined with a biphasic or broad peak between 250 and 280 nm may indicate contamination with phenol rather than chaotropic salts. These contaminants may have a negative impact on the efficiency of the protocol.

Genomic DNA Contamination

Depending on the RNA extraction protocol used, samples may also contain significant amounts of genomic DNA (gDNA), which is indistinguishable from RNA on a spectrophotometer. Furthermore, as many of the dyes used in RNA microfluidics assays stain single-stranded nucleic acids much more intensely than double-stranded, low to moderate amounts of gDNA may not be readily visible with an RNA-specific microfluidics assay. We highly recommend examining all RNA samples on a denaturing agarose gel or using a fluorometric assay with DNA- and RNA-specific dyes to check samples for DNA contamination. On an agarose gel gDNA can appear as either a dark mass, which remains in the slot if relatively intact or as a high molecular weight smear if it has been sheared during extraction.

The best way to avoid gDNA contamination is to use an RNA extraction protocol that does not co-isolate gDNA (e.g., Lexogen's SPLIT RNA Extraction Kit, Cat. No. 008.48). However, DNA can be removed from irreplaceable samples by acidic phenol extraction or DNase I digestion. We do not recommend DNase treatment, as the extended incubation with divalent cations can lead to RNA hydrolysis and decrease RNA integrity. If samples must be DNase treated, heat inactivation should be avoided, and the enzyme should be deactivated by other means such as phenol/chloroform extraction or silica column purification.

RNA Integrity Check

The integrity of an RNA sample can be assessed with a variety of methods. We recommend the use of a microfluidics assay such as the RNA6000 series for the 2100 Bioanalyzer (Agilent Technologies, Inc.), although RNA quality can also be assessed with denaturing agarose gel electrophoresis if such a device is not available. Most microfluidics platforms will carry out an automated peak analysis and generate a quality score (RIN or RQN). RNA integrity should also be taken into consideration when designing your target-specific primers. Choose your targeted region accordingly (see also Appendix D, p.28).

Mitochondrial Ribosomal RNA

Mitochondrial ribosomal RNAs (mt-rRNAs) are polyadenylated and hence will also be reverse transcribed and converted into a cDNA library if oligodT priming is used during First Strand Synthesis. mt-rRNAs can make up 1 - 2 % of the reads when using a 3' mRNA Seq protocol. Optionally, an rRNA depletion method, which also removes mt-rRNAs, such as Lexogen's RiboCop rRNA Depletion Kit (Cat. No. 037) can be used before starting the QuantSeq library preparation.

RNA Storage

If immediate RNA extraction is not possible, tissue samples can be either flash-frozen with liquid nitrogen or submerged in RNAlater (Life Technologies, Inc.) and stored at -80 °C. After extraction, RNA can be stored at -20 °C or -80 °C in 10 mM Tris pH 7.0. Addition of RNasin or an equivalent RNase inhibitor is recommended. Avoid frequent freeze/thaw cycles as RNA might get sheared.

SIRVs Spike-In RNA Variant Control Mixes

Lexogen offers a set of artificial spike in transcripts called SIRVs (Spike-In RNA Variants), to serve as a control and anchor set for the comparison of RNA-Seq experiments. The SIRVs consist of 69 artificial RNA transcripts with no identity to any known genomic sequences, hence they can be spiked into any RNA. SIRVs are available in three sets, SIRV-Set 1 (Cat. No 025) contains the Isoform Mixes E0, E1, and E2 of the isoform module, SIRV-Set 2 (Cat. No. 050) provides the Isoform Mix E0 only, whereas SIRV-Set 3 (Cat. No. 051) has the SIRV Isoform Mix E0 in a mixture with the ERCC RNA Spike-in controls (Ambion Inc., see below). The SIRVs are polyadenylated mRNAs and therefore are efficiently captured during QuantSeq 3' library preparation.

ERCC RNA Spike-in Controls

To enable the hypothesis-neutral calculation of strandedness, to assess internal oligodT priming events, and as a true reference on detection limit and preservation of dynamic range, we highly recommend the addition of artificial transcripts of known strand orientation and concentration such as the ERCC RNA Spike-in controls (Ambion Inc.). For QuantSeq we recommend using SIRV-Set 3 (Cat. No. 051), which contains ERCCs together with the SIRV isoform controls. ERCCs have a known strand orientation and no antisense transcripts, so the calculation of strandedness based on ERCC sequences is more accurate than calculations based on reads aligned to the genome. The input-output correlation can be computed by comparing the given concentrations of the ERCC RNA Spike-in transcripts with their expression value in the sequenced library. Any potential overcycling of the libraries can be detected. Transcripts may have different and not yet annotated 3' ends, which might be mistaken for internal priming events of the oligodT primer, when in fact those are true 3' ends. As ERCC transcripts only have one defined 3' end, this provides the only true measure to determine internal priming.

ATTENTION: If spike-in transcripts are used for targeted sequencing approaches, primers specifically targeting the spike-in transcripts of interest need to be designed (for general information on primer design see Appendix D, p.28).

8. Appendix B: RNA Input Quality and Amount

The amount of total RNA required as input for QuantSeq depends on the poly(A) RNA content of the sample in question and RNA quality. In general, high quality mRNA-Seq data relies on high quality input RNA. Especially when using targeted sequencing during first strand and second strand synthesis, the RNA quality becomes important. However, as QuantSeq is a 3' mRNA-Seq protocol both high and low quality RNA can be used as input.

Input Guidelines

- The minimum recommended input amount for the QuantSeq-Flex library prep is 10 ng total RNA.
- The maximum input for the protocol is 2 µg.
- As a starting point, we recommend performing the protocol initially with 100 - 500 ng total RNA. RNA inputs ≥200 ng are recommended to detect low abundant transcripts efficiently.
- QuantSeq-Flex is also highly suitable for low quality, degraded, and FFPE samples and require some protocol modifications (Appendix D, p.28). For low quality RNA, the targeted region should be kept small (e.g., 100 - 200 bp) so both primers are still able to bind to the RNA fragments. By using targeted priming only for one of the cDNA synthesis steps e.g., targeted primers only during first strand synthesis and random priming during second strand synthesis, also lower quality RNA can be used as input material. For low quality RNA also oligodT priming during first strand synthesis and targeted primers during second strand synthesis can be used.
- As targeted sequencing depends on the primers used and the abundance of the target RNAs in the tissue in question, optimal cycle numbers need to be determined by qPCR (for details see Appendix F, p.31).
- For increasing the specificity of the reaction using custom primers, protocol adjustments such as increased reaction temperatures may be beneficial.

QuantSeq with oligodT-primed first strand synthesis and random primed second strand synthesis (standard protocol) was extensively tested with various cell cultures, mouse and plant tissues, yeast, fungi, and human reference RNA (Universal Human Reference RNA (UHRR) and Human Brain Reference RNA (HBRR)). For further information please refer to the QuantSeq 3'mRNA-Seq User Guide (015UG009).

9. Appendix C: Low Quality RNA - FFPE

RNA isolated from Formalin-Fixed Paraffin Embedded (FFPE) samples is often heavily degraded. QuantSeq with oligodT priming is highly suitable for FFPE RNA, as the fragments are generated near the 3' end. For QuantSeq-Flex it is recommended to keep the targeted region as short as possible (between 100 bp - 200 bp) in order to account for the degraded RNA starting material. For FFPE samples only minor protocol adjustments are required, specifically

- Skipping step 2 for oligodT primed reverse transcription
- Reducing the addition of **PS** in step 16 to 48 µl.
- Using only 27 µl **PB** in step 29 for single indexing PCR, and 31.5 µl in step 30 for dual indexing PCR, respectively (see FAQs at www.lexogen.com).

Further optional adjustments for low input FFPE RNA samples (<10 ng) may also be included, such as:
Extending the reverse transcription time in step 4 to 1 hour.

In addition to the RNA Integrity Number (RIN), the quality of FFPE RNA is assessed by the DV_{200} value. The DV_{200} value is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV_{200}, the more degraded the RNA is. Please keep the level of degradation in mind when choosing your custom targeted primers. Especially when using targeted sequencing during first strand and second strand synthesis, the RNA quality becomes extremely important. For extremely degraded samples we recommend using targeted priming only for one of the cDNA synthesis steps e.g., targeted primers only during first strand synthesis and random priming during second strand synthesis. For low quality RNA also oligodT priming during first strand synthesis and targeted primers during second strand synthesis can be used. However, take into consideration that your targeted second strand synthesis primers should be located near the 3' end (optimal within 100 - 200 bases upstream of the poly(A) tail.

ATTENTION: To determine the exact cycle number of your endpoint PCR, we strongly recommend using the PCR Add-on Kit (Cat. No. 020) and **taking advantage of the qPCR assay** as described in Appendix F, p.31.

ATTENTION: FFPE RNA is degraded RNA and hence the insert sizes are smaller than for non-degraded RNA samples. Keep this in mind when choosing your sequencing length.

If you see that your FFPE RNA still generates a lot of linker-linker products, an additional purification of the lane mix with 0.9 x **PB** (e.g., 50 µl lane mix plus 45 µl **PB**), incubating 5 minutes at room temperature, and following the protocol from step 30 on again may be necessary.

10. Appendix D: Primer Design

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 purification step (step 12 and 16). The full Illumina P7 (Read 2) adapter sequence will only be introduced during PCR (step 28).

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).

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 (25 nt Illumina-sequence + 20 - 25 nt targeted sequence). Optionally, a 6 - 8 nt long molecular index (NNNNNN(NN)) between adapter sequence and target sequence could also be included, but then a paired-end sequencing run is required for read-out. With molecular indices, PCR duplication events can be distinguished from unique priming events.

We highly recommend checking your 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 RNA 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 purification step (step 12 and 16).The full Illumina P5 (Read 1) adapter sequence will only be introduced during PCR (step 28).

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.

REMARK: Introduction of 1 - 3 phosphorothioate linkages (PTOs) at the 3' end of the target-specific second strand synthesis primer may increase specificity.

We highly recommend checking your 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.

11. Appendix E: Primer Concentrations

First Strand Synthesis

The concentration of a target-specific first strand synthesis primer should be around 12.5 nM - 1.25 µM final concentration (i.e., 5 µl of a 50 nM - 5 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.

Second Strand Synthesis

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.

12. Appendix F: qPCR

The mRNA content and quality of total RNA affects the number of PCR cycles needed for the final library amplification step. We strongly recommend taking advantage of the qPCR assay. Over- or undercycling may bias your sequencing results (transcript abundance estimation and library quantification) and can be avoided by optimising the PCR cycle number.

qPCR to Determine the Optimal Cycle Number for Endpoint PCR

The PCR Add-on Kit for Illumina (Cat. No. 020) provides additional PCR Mix (**PCR** ●), Enzyme Mix (**E** ●) , and the P7 Primer (**7000** ●) needed for the qPCR assay. In addition, SYBR Green I nucleic acid dye (Sigma Aldrich, S9430, user-provided) is also required for quantification.
ATTENTION: The use of SYBR Green I-containing qPCR mastermixes is not recommended.
NOTE: SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines have 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 (H_2O).
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 the 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 (**H_2O**). **ATTENTION:** Include a no template control!
4	Perform 40 - 50 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, 45 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, hold at 10 °C **REMARKS:** 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.

REMARK: The qPCR assay can be used for dual- or single-indexed libraries.

Example for Endpoint Calculation

500 ng total RNA input was used for generating libraries. Using 1.7 µl of cDNA for a qPCR, the cycle number corresponding to 50 % of the maximum fluorescence was 15 cycles. The remaining 17 µl of the template should therefore be amplified with 12 cycles (15-3 cycles =12 cycles).

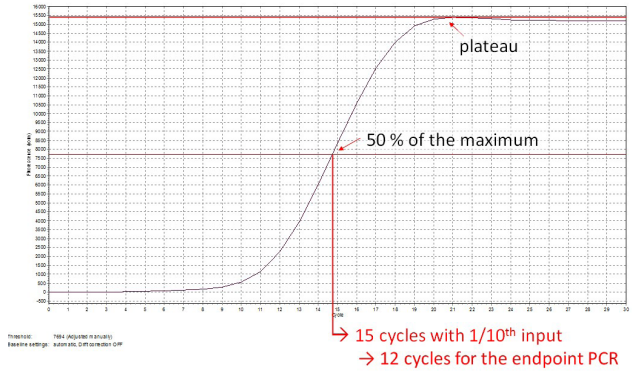

Figure 4. Calculation of the number of cycles for the endpoint PCR.

NOTE: Once the number of cycles for the endpoint PCR is established for one type of sample (same input amount, tissue, and RNA quality), there is no need for further qPCRs. The entire cDNA can be inserted straight into the endpoint PCRs.

13. Appendix G: Library Reamplification

Reamplification of Single-Indexed Libraries (i7 only)

Lexogen's PCR Add-on Kit also 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. 020.96) Instruction Manual.

Reamplification of Dual-Indexed Libraries (i5 and i7)

For reamplification of dual-indexed libraries the Reamplification Add-on Kit for Illumina (Cat. No. 080.96) is available on request. Please contact Lexogen at info@lexogen.com.

14. Appendix H: Library Quality Control

Quality control of finished QuantSeq libraries is highly recommended and can be carried out with various methods depending on available equipment. A thorough quality control procedure should include the analysis of both the concentration and the size distribution of libraries.

Quality Control Methods

The analysis of a small volume of the amplified library with microcapillary electrophoresis has become the de facto standard 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 Agilent Bioanalyzer 2100 and High Sensitivity DNA chips (Agilent Technologies, Inc.). For high throughput applications instruments such as the Fragment Analyzer (Advanced Analytical Technologies, Inc.), LabChip GX II (Perkin Elmer), or 2200 TapeStation (Agilent Technologies, Inc.) are recommended. Typically, 1 µl 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 µl of the finished library may be diluted to the required volume (e.g., 2 µl sample for TapeStation and 10 µl 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 Cq 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. Most UV-Vis spectrophotometers are not sensitive enough at these concentrations to accurately quantify NGS libraries and should be avoided.

15. Appendix I: Typical Results

QuantSeq-Flex libraries are intended for a high degree of multiplexing, hence libraries do not need to be extensively amplified. Best practice is to use the qPCR for the determination of the endpoint PCR cycle number (see Appendix F, p.31). This will prevent overcycling and distorting expression values while at the same time providing enough material for quantification and subsequent cluster generation. Cycle numbers and libraries generated depend on the primers and the RNA input material used.

REMARK: QuantSeq-Flex libraries with oligodT priming during first strand synthesis and random priming during second strand synthesis generate a different library profile (longer insert length) than QuantSeq FWD (Cat. No. 015) libraries (see Figure 5). The difference in insert length is caused by the different volumes and protocol used during first strand synthesis (see also Appendix J, p.36). Typical concentrations of QuantSeq-Flex control libraries (oligodT primed first strand synthesis and random primed second strand synthesis) are between 6 - 11 nM (1.2 - 2.3 ng/µl) for 500 ng (12 cycles) Universal Human Reference RNA (UHRR) input, respectively.

A shorter side-product caused by priming of the second strand synthesis oligo on the oligodT or targeted primer is sometimes visible at ~140 bp, and should not compose more than 0 - 3 % of the total library. Higher proportions of this side-product can indicate problems during library preparation. Low input RNA for instance will result in an increase of this side-product. For removal of side-products, see p.27.

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. To prevent over- or undercycling of libraries, we recommend using the qPCR assay as described in Appendix F, p.31. For targeted priming approaches the qPCR assay is of particular importance, as the abundance of the targeted transcripts determines the amount of PCR cycles required during the amplification.

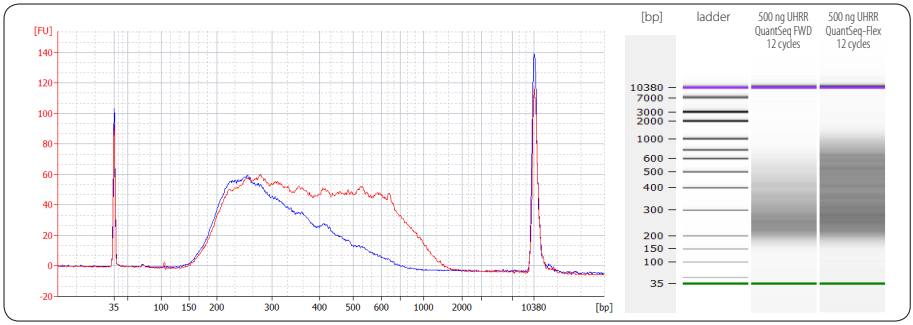

Figure 5. Bioanalyzer traces of QuantSeq-Flex (red trace) with oligodT primed first strand synthesis (Cat. No. 026) and random primed second strand synthesis vs QuantSeq FWD (Cat. No. 015) libraries (blue trace) synthesized from 500 ng total RNA input, amplified with 12 cycles. Input RNA was Universal Human Reference RNA (UHRR).

Examples for target-specific priming are depicted in Figure 6. Primers were designed against B-Raf proto-oncogene, serine/threonine kinase (BRAF, NM_004333.4), erb-b2 receptor tyrosine

kinase 2 (ERBB2, NM_001005862.2), and KRAS proto-oncogene, GTPase (KRAS, NM_004985.4) and used with QuantSeq-Flex on K562 total RNA. An RNA input amount series (500 ng, 100 ng, and 10 ng K562 RNA) for the detection of BCR-ABL fusion transcripts is depicted in Figure 7.

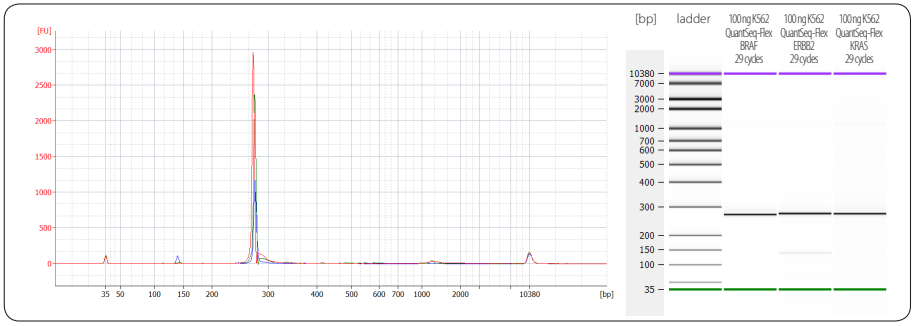

Figure 6. Bioanalyzer traces of QuantSeq-Flex libraries with target-specific primers for BRAF (NM_004333.4, red trace), ERBB2 (NM_001005862.2, blue trace), and KRAS (NM_004985.4, green trace) transcripts. 100 ng K562 total RNA was used as input material. First strand synthesis was performed at 50 °C using the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026) and 1.25 µM final RT primer concentration (BRAF RT primer: 5' GTTCAGACGTGTGCTCTTCCGATCTCAAAATGGATCCAGACAACTGTTCA 3'; ERBB2 RT primer: 5' CACGACGCTCTTCCGATCTNNNNNNGCCATCACGTATGCTTCGTCTA 3', and KRAS RT primer: 5' GTTCAGACGTGTGCTCTTCCGATCTGCACTGTACTCCTCTTGACCTG 3'). RNA removal was skipped and second strand synthesis was performed with the QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028) and 0.5 µM final concentration of a target-specific second strand synthesis primer with annealing at 65 °C for 60 seconds and extension at 72 °C for 5 minutes (BRAF SSS primer: 5' CACGACGCTCTTCCGATCTNNNNNNGCCAAGTCAATCATCCACAG*A*G*A 3', ERBB2 SSS primer: 5' GTTCAGACGTGTGCTCTTCCGATCTGCTTGGATCTGGCGCTTTT 3', and KRAS SSS Primer: 5' CACGACGCTCTTCCGATCTNNNNNNGAGTGCCTTGACGATACAG*C*T*A 3'). All three libraries were amplified for 29 cycles. A 6 nucleotide long molecular index (NNNNNN) was used either with the RT primer (ERBB2, here with P7 linker, blue trace) or the second strand synthesis primer (BRAF, red trace and KRAS, green trace, with P5 linker). The peak at 272 bp corresponds to the targeted region: 150 bp for BRAF, ERBB2, and KRAS, respectively, the 6 nt molecular index, and the 116 bp from the Illumina P5 and P7 adapter sequences. If a multiplexing index would be used, the Illumina adapter sequences would be 122 bp. For ERBB2 (blue trace) a minor peak at 140 bp is visible, which corresponds to a primer artifact (target-specific primers plus Illumina-extensions). High abundances of such side-products may influence the qPCR endpoint determination negatively. Increase RNA input amount if the side-product is too prominent. * indicate phosphorothioate linkages (PTOs) to increase specificity

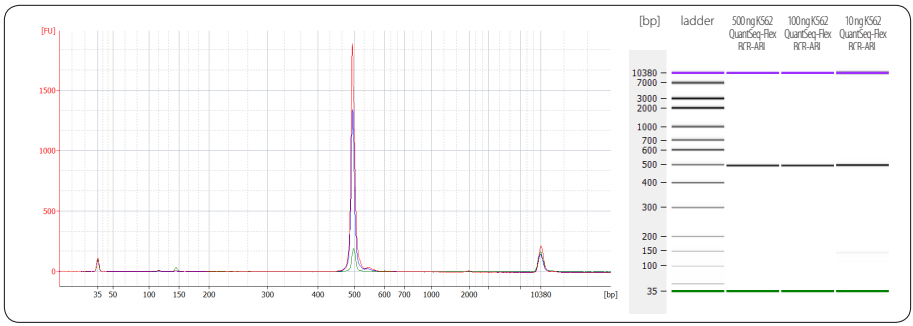

Figure 7. Bioanalyzer traces of QuantSeq-Flex libraries with target-specific primers for BCR-ABL fusion transcripts. First strand synthesis was performed at 50 °C using the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026) and 12.5 nM final concentration of a targeted primer (ABL-a2: 5' CGTGTGCTCTTCCGATCTTGTTGACTGGCGTGATGTAGTTGCTTGG 3'). The QuantSeq-Flex Second Strand Synthesis Module V2 (Cat. No. 028) and 0.5 µM final concentration of the target-specific BCR-b2 primer (5' CACGACGCTCTTCCGATCTACAGAATTCCGCTGACCATCAATAAG 3') was used as described on p.15 with annealing at 68 °C. 500 ng (red trace, 24 cycles), 100 ng (blue trace, 26 cycles), and 10 ng (green trace, 28 cycles) K562 total RNA was used as input material. At 10 ng total RNA input a side-product at 145 bp becomes visible. High abundances of such side-products may influence the qPCR endpoint determination negatively. Increase RNA input amount if the side-product is too prominent.

16. Appendix J: Size Selection

The QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026) can be used to increase insert sizes. For details regarding oligodT primed first strand synthesis using the QuantSeq-Flex First Strand Synthesis Module, please refer to the corresponding User Guide.

In short: **FS1** ● and **FS2** ● from the standard QuantSeq FWD Kit are exchanged with **FS1x** ●, **FS2x** ●, and **dT** ● from the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026). Due to buffer differences and protocol changes longer inserts can be generated.

Examples of such library length increases are depicted in Figure 7.

Step 2 (3 min 85 °C) Contains	Mastermix Added in Step 4	Library*			Insert				Library Yield		PCR Cycles
		Start [bp]	End [bp]	Mean Size*	Mean Size	≥ 50 nt	≥ 100 nt	≥ 200 nt	ng/µl	nM	
RNA + FS1	FS2/E1	132	9,000	381	249	95 %	62 %	17 %	3.3	18.1	13
RNA + dT	FS1x/FS2x/E1	132	9,000	491	359	96 %	74 %	33 %	3.1	14.4	13
RNA + FS1x + dT	FS2x/E1	132	9,000	424	292	96 %	72 %	28 %	2.7	13.2	13

*All libraries were prepared from 500 ng Universal Human Reference RNA (UHRR) with dual indexing. Linker sequences are 132 bp including 6 nt long i5 and i7 indices, respectively. FS1x, FS2x, and oligo dT (dT) are components from the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026). FS1 and FS2 are from the standard QuantSeq FWD Kit.

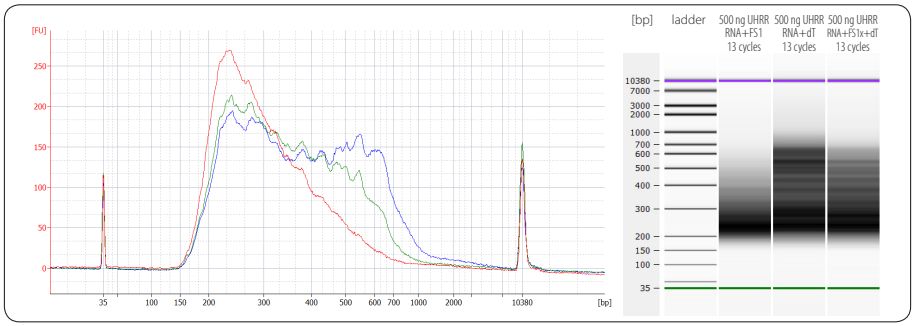

Figure 8. Bioanalyzer traces of QuantSeq FWD HT libraries with library size modulation. The shortest insert sizes are generated with the standard QuantSeq Kit components, i.e., denaturing of RNA in FS1 (red trace, standard protocol). With the QuantSeq-Flex First Strand Synthesis Module and denaturing RNA and oligodT only, before adding FS1x, FS2x, and E1 the longest average insert sizes are generated (dark blue trace). With the QuantSeq-Flex First Strand Synthesis Module and denaturing RNA, FS1x, and dT before adding FS2x and E1 the average insert size can be increased (green trace). Input RNA was 500 ng Universal Human Reference RNA (UHRR) for all libraries. Dual indexing with 13 cycles of final library amplification was used for all libraries.

17. Appendix K: Unique Molecular Identifiers

Unique Molecular Identifiers (UMIs) can be included in QuantSeq-Flex libraries to enable the detection and removal of PCR duplicates.

Target-Specific Reverse Transcription (Random Second Strand Synthesis)

The UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1) (Cat. No. 081) can only be used for random primed second strand synthesis. It includes the UMI Second Strand Synthesis Mix (**USS** ●), which contains UMI-tagged random primers. The **USS** ● simply replaces the Second Strand Synthesis Mix 1 (**SS1** ●) from the standard QuantSeq FWD Kit. No other protocol changes are required. The UMIs are added between the partial P5 adapter and the random priming sequence, during second strand synthesis (Fig. 10).

Short Protocol - Second Strand Synthesis

NOTE: This protocol replaces steps 7 and 8 of the detailed protocol from the QuantSeq-Flex RNA-Seq Library Prep Kit for Illumina User Guide (p.17). Step 8 has not been changed for UMI libraries and is included here for ease of reference.

	Follow steps 1 - 6 as indicated in the detailed protocol (p.13-17).
7	Add 10 µl of UMI Second Strand Synthesis Mix (**USS** ●) to the reaction. Mix well by pipetting, and seal the plate. **REMARK:** Use a pipette set to 30 µl for efficient mixing.
8	Incubate the plate for 1 minute at 98 °C in a thermocycler, and slowly cool down to 25 °C by setting the ramp speed to 10 % (0.5 °C/second). Incubate the reaction for 30 minutes at 25 °C. Quickly spin down the plate at room temperature before removing the sealing foil.
➡	Proceed to step 9 of the detailed protocol (p.17).

Sequencing

A minimum length of 75 bp (i.e., SR75 or longer) is recommended for sequencing QuantSeq libraries that include UMIs. The 6 nt UMI is read-out at the beginning of Read 1, upstream of the random priming sequence (Fig. 9). QuantSeq libraries containing UMIs are compatible with dual indexing using the i5 Dual Indexing Add-on Kits for QuantSeq/SENSE (5001-5096) (Cat. No. 047). No custom sequencing primers are required.

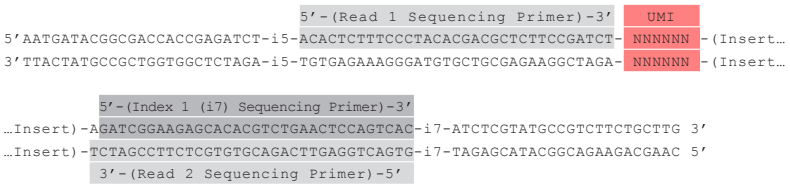

Figure 9. QuantSeq FWD libraries with UMIs.

Data Analysis

Sequencing data containing UMIs can be analyzed using the QuantSeq Data Analysis pipeline available on the Bluebee® Genomics Platform. An additional command-line analysis tool (**collapse_UMI_bam)** is also available for de-duplication of sequencing read counts using UMIs. To obtain a copy of the source code, or for further information on UMI data analysis methods, please contact info@lexogen.com.

Target-Specific Second Strand Synthesis (Random Reverse Transcription)

When designing the target-specific primers 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. Please refer to Appendix D, p.28 for more information.

18. Appendix L: Multiplexing

QuantSeq libraries are designed for a high degree of multiplexing. The i7 indices represent the minimum requirement for sample indexing for multiplexed sequencing and are added during the PCR amplification step. 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 (i7 Index Plate, 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: AACGCC	7034: TTGGTA	7042: TGACAC	7050: TCGAGG	7058: ATAAGA	7066: GACGAT	7074: CGATCT	7082: ACTCTT	7090: CCTGCT
C	7003: ACCAGT	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

i7 index sequences are available for download at www.lexogen.com.

In general, we recommend processing a minimum of 8 samples, using a complete set of eight i7 indices for multiplexing (e.g., **7001-7008**). However, if fewer indices are required care should be taken to always use indices which 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, e.g., if QuantSeq libraries are multiplexed with 8 nt indexed libraries from other NGS-kit suppliers, 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 Indices

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

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

The i5 Dual Indexing Add-on Kit for QuantSeq/SENSE (5001–5004, Cat. No. 047.4) provides four different i5 index primers (5001–5004). Each tube contains sufficient volume for preparing 24 (Cat. No. 047.4×24), or 96 (Cat. No. 047.4×96) libraries. This kit is therefore ideal for combinatorial dual indexing strategies (see FAQ 5.6). In combination with 96 i7 indices, a maximum of 384 (4 i5 x 96 i7) dual-indexed libraries with unique i5 / i7 index combinations can be multiplexed in a single sequencing lane or run.

Lane Mix Repurification to Remove Linker-Linker Artifacts

A shorter side-product representing linker-linker artifacts is sometimes visible at ~150 bp, 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).

1	Measure the volume of the library or lane mix. If the volume is less than 20 µl, adjust the total volume to 20 µl using Elution Buffer (**EB**) or molecular biology-grade water (**H_2O**).
2	Add 0.9 volumes (0.9x) of Purifiction 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.20-21).

19. Appendix M: 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 at www.lexogen.com under QuantSeq Frequently Asked Questions (FAQs 1.24 (FWD)).

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, QuantSeq-Flex or SENSE 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 Frequently Asked Questions (FAQs).

QuantSeq FWD and QuantSeq-Flex Libraries with i7 Indices

i7 Indices (6 nt) are introduced during PCR (step 28). For QuantSeq FWD and QuantSeq-Flex libraries, Read 1 directly corresponds to the RNA sequence.

```
                                5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert…
3'TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert…

                 5'-(Index 1 (i7) Sequencing Primer)-3'
   …Insert)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
   …Insert)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
                  3'-(Read 2 Sequencing Primer)-5'
```

Read 1: Multiplexing Read 1 Sequencing Primer (not supplied):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

Index 1 Read (i7): Multiplexing Index 1 Sequencing Primer (not supplied):
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied):
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

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

A schematic representation of the QuantSeq FWD and QuantSeq-Flex libraries (Cat. No. 015 and Cat. No. 026, 028, 033, 034, 035) is shown above. If a molecular barcode (see Appendix D, p.28) was introduced for QuantSeq-Flex targeted second strand synthesis, the random sequence corresponding to the molecular barcode is found at the beginning of Read 1 (i.e., between Read 1 sequencing primer and target sequence/insert). If a molecular barcode was introduced during first strand synthesis, a paired-end read out is required and the random sequence will be located at the beginning of Read 2 (i.e., between Read 2 sequencing primer and target sequence/insert).

ATTENTION: We do not recommend paired-end sequencing for oligodT primed libraries, as the quality of Read 2 would be very low due to the poly(T) stretch at the beginning of Read 2.

20. Appendix N: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of QuantSeq data and is kept as general as possible for integration with your standard pipeline.
QuantSeq FWD (Cat. No. 015) and QuantSeq-Flex (Cat. No. 026, 028, 033, 034, and 035) kits contain 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 and Read 1 directly reflects the mRNA sequence.

Demultiplexing

i5 and i7 indices: Demultiplexing can be carried out by the standard Illumina pipeline. Index sequences are available for download at www.lexogen.com.

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.

Alignment

The filtered and trimmed reads can be aligned with a short read aligner to the reference genome. STAR aligner or TopHat can be used for mapping QuantSeq FWD and QuantSeq-Flex data.

Integrated Data Analysis Pipeline at Bluebee

Each purchased QuantSeq kit includes a code for free data analysis including differential expression (DE) analysis using the Bluebee platform. The data analysis pipeline is only set up for the standard QuantSeq FWD and REV Kits, please contact info@lexogen.com if you want to use Bluebee for your QuantSeq-Flex data analysis.

21. Appendix O: Revision History

Publication No.	Change	Page
015UG058V0230 Aug. 8, 2018	Pre-warm mastermixes for first-strand synthesis (step 3).	13-15
	After denaturing (step 2), leave the reactions on the thermocycler at 42 – 50 °C until step 4.	13-15
	Information on the handling of low quality, degraded, FFPE samples for first-strand synthesis added (steps 1-4).	13-15
	Safe stopping point removed after step 4: Proceed immediately to RNA removal.	13, 14
	Information on UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1, Cat. No. 081) added.	17
	Restructuring Appendix F (qPCR) and new Appendix G (Library Reamplification).	31, 32
	Addition of Appendix J (Modulating Insert Sizes) and Appendix K (UMIs).	36-38
	Shortened Revision History (only 2016 onwards).	43
015UG058V0220 Feb. 7, 2017	Included new QuantSeq FWD basic kit protocol (only RS, new SS1, less PB post SSS).	7, 16, 17
	Barcode plate (BC) was rearranged for improved balance and renamed to i7 Index Plate (7001-7096). Previous BC05: TAATCG replaced by 7025: TTTATG to avoid overlap with Illumina-specific indices.	7, 18, 32
	Barcode 00 (BC00) in PCR Add-on Kit renamed to P7 Primer 7000.	28
	qPCR endpoint determination using only 1.7 µl.	28
	Evaluation tool to check the color balance of index subsets.	32
015UG058V0200 Oct. 19, 2016	New streamlined protocol for target-specific second strand synthesis.	4, 6, 15, 20, 31
	Indication of safe stopping points.	13 - 20
	Increased insert size for 3' mRNA-Seq libraries with QuantSeq-Flex First Strand Synthesis Module.	13 - 14, 30
	Restructuring of Appendices (separate Appendices on qPCR and Typical Results).	24 - 35
	NCBI Primer Blast tool for Primer Pair Specificity Check.	25, 26
	qPCR endpoint determination now set to 50 % of the maximum fluorescence and using less template for qPCR.	28
	Typical Results for BRAF, ERBB2, and KRAS targeted primining in K562 RNA (Fig. 5).	30 - 31
	Input series for K562 RNA and BCR-ABL fusion transcript detection (Fig. 6).	31
015UG058V0110 Mar. 29, 2016	Changes in denaturation procedure for targeted priming during RT.	13, 20
	RT temperature set to at least 42 °C.	14, 20
	SS1 dilutions for longer insert sizes. Only valid for random primed SSS.	16, 20
	Increased qPCR cycle number + NTC for low RNA input, low abundant targets.	22
	ERCC and SIRV Spike-in Mixes.	24
	Molecular barcodes in RT primer (requires PE sequencing run for read-out).	25
015UG058V0100 Jul. 15, 2015	Initial Release.	

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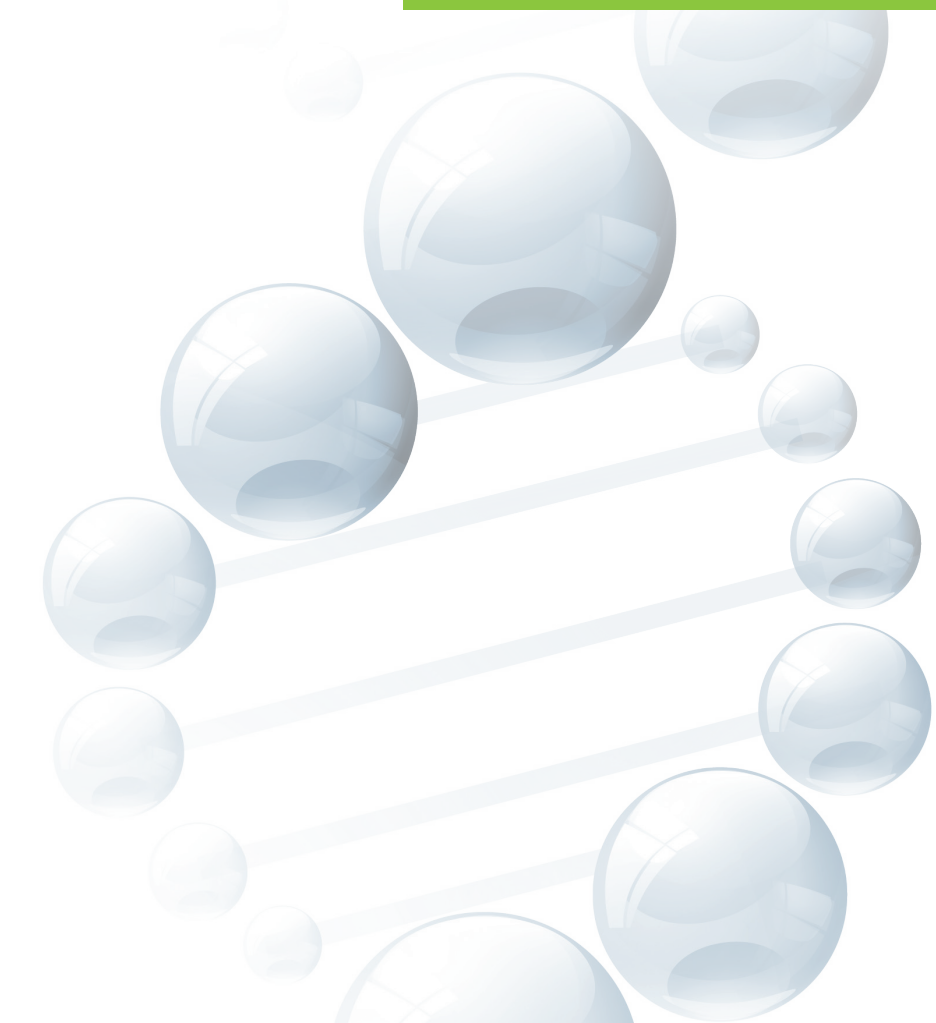

QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2 · User Guide

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