

QUANTTM SEQ

Sequencing that counts

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

Catalog Numbers:

- 015 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (FWD))
- 016 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (REV) with Custom Sequencing Primer)
- 020 (PCR Add-on Kit for Illumina)
- 022 (Purification Module with Magnetic Beads)
- 026 (QuantSeq Flex First Strand Synthesis Module compatible with QuantSeq 015)
- 028 (QuantSeq Flex Second Strand Synthesis Module compatible with QuantSeq 015)
- 033 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit with First Strand Synthesis Module)
- 034 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit with Second Strand Synthesis Module)
- 035 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit with First and Second Strand Synthesis Modules)

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

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1. Overview

Lexogen's QuantSeq kit is now also available with flexible modules for First Strand and/or Second Strand Synthesis allowing the use of custom primers. This also 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, no matter 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 a maximum flexibility. Reverse transcription can be primed with an oligodT primer (dT, included in the kit) or target-specific primers (to be provided by the user). When designing targeted primers, please be advised to include the Illumina P7 sequence (see Appendix D, p.26) at the 5' end of your reverse transcription primers. Second strand synthesis can either be initiated by random priming simply by using SS1 from QuantSeq 015 or target-specific primers (to be provided by the user). Include the Illumina P5 sequence (see Appendix B, p.26) at the 5' end of your second strand synthesis primers.

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

- 1.) OligodT primed during RT, random primed during SSS (QuantSeq 3' mRNA-Seq)
- 2.) OligodT primed during RT, target-specifically primed during SSS (targeted 3' mRNA-Seq)
- 3.) Target-specifically primed during RT, random primed during SSS (targeted RNA-Seq, allows for identification of novel fusions)
- 4.) Target-specifically primed during RT, target-specifically primed during SSS (targeted RNA-Seq, known targets detectable only)

QuantSeq maintains strand-specificity and allows mapping of reads to their corresponding strand on the genome, enabling the discovery and quantification of antisense transcripts and overlapping genes. The kit includes magnetic beads for the purification steps and hence are compatible with automation. Multiplexing of libraries can be carried out using up to 96 external barcodes.

QuantSeq uses total RNA as input, hence no prior poly(A) enrichment or rRNA depletion is required, although if using random priming for reverse transcription a depletion may be advisable. Information regarding input RNA requirements can be found in Appendix A (p.22).

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 G, p.32, for a schematic representation of the finished library).

External barcodes are included in the QuantSeq kits and are introduced during the PCR amplification step (Appendix F, p.31).

Library quantification can be performed with standard protocols and is further discussed in Appendix E (p.29).

Libraries are compatible with Single-End and Paired-End Sequencing. However, for library option 1.) and 2.) we do not recommend paired-end sequencing as Read 2 would start with the poly(T) stretch and the quality of sequencing reaction would therefore be low. When using targeted primers for second strand synthesis please keep consider the requirements for cluster calling on Illumina platform (see Appendix B, p.26)

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 H (p.33).

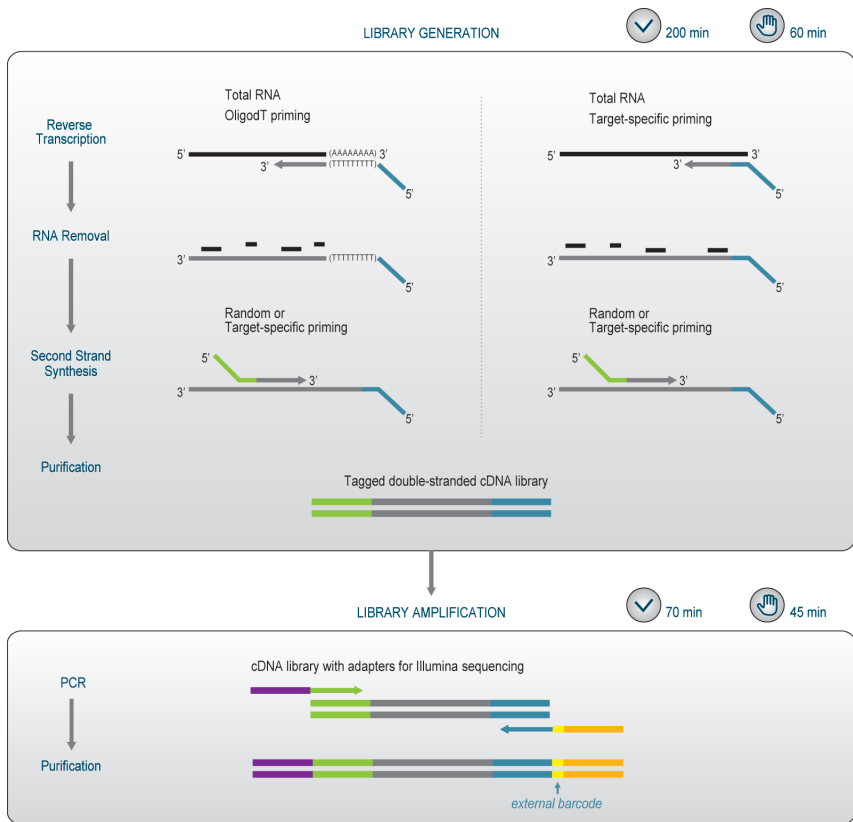


Figure 1. Schematic overview of the QuantSeq-Flex Targeted RNA-Seq library preparation workflow. Read 1 reflects the RNA sequence. The reverse transcription reaction can either be primed using an oligodT primer (included in the kit) or a target-specific primer (not included, custom designed for desired 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). Depending on the combination of different priming options 4 different libraries can be generated.

2. Kit Components and Storage Conditions

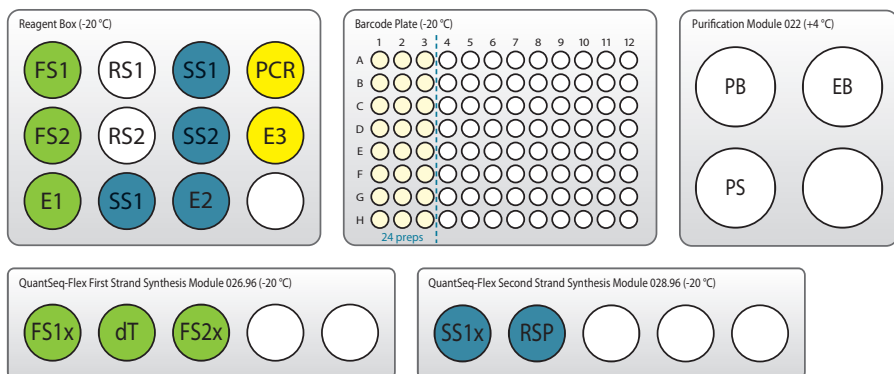


Figure 2. Location of kit components. QuantSeq-Flex First Strand Synthesis Module can be used to substitute FS1 and FS2 from QuantSeq 015 FWD, allowing to add a custom primer. An oligodT primer is included as control. QuantSeq-Flex Second Strand Synthesis Module can be used to substitute SS1 from QuantSeq 015 FWD, allowing to add a custom primer. A random primer is included as control.

QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026)	Tube Label	Volume* needed for 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 ●	475.2 µl	-20 °C

*including a 10 % surplus

QuantSeq-Flex Second Strand Synthesis Module (Cat. No. 028)	Tube Label	Volume* needed for 96 preps	Storage
QuantSeq-Flex Second Strand Synthesis Mix 1	SS1x ●	1584 µl	-20 °C
Random Second Strand Synthesis Primer	RSP ●	212 µl	-20 °C

*including a 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.8).

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 can be used with the First Strand Synthesis components included in the QuantSeq 015 kit, depending what kind of libraries are to be generated.

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 B, p.26). Alternatively include a random sequence of 5 - 8 nt between linker sequence and target sequence.

Kit Component (Cat. No. 015) QuantSeq 015 FWD Reagent Box	Tube Label	Volume* needed for	Storage
		96 preps	
First Strand cDNA Synthesis Mix 1	FS1 ●	528 µl	-20 °C
First Strand cDNA Synthesis Mix 2	FS2 ●	1003.2 µl	-20 °C
Enzyme Mix 1	E1 ●	52.8 µl	-20 °C
RNA Removal Solution 1	RS1 ○	528 µl	-20 °C
RNA Removal Solution 2	RS2 ○	528 µl	-20 °C
Second Strand Synthesis Mix 1	SS1 ●	1584 µl	-20 °C
Second Strand Synthesis Mix 2	SS2 ●	422.4 µl	-20 °C
Enzyme Mix 2	E2 ●	105.6 µl	-20 °C
PCR Mix	PCR ●	739.2 µl	-20 °C
Enzyme Mix 3	E3 ●	105.6 µl	-20 °C
Barcode Plate (96-well plate)	BC	5 µl / reaction	-20 °C
Purification Module (Cat. No. 022) included in the kit			
Purification Beads	PB	5280 µl	+4 °C
Purification Solution	PS	10771 µl	+4 °C
Elution Buffer	EB	11616 µl	+4 °C

*including a 10% surplus

Upon receiving the QuantSeq kit, store the Purification Module (Cat. No. 22.96), containing **PB**, **PS**, and **EB**) at +4 °C and the rest of the kit in a -20 °C freezer.

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

3. User-supplied Consumables and Equipment

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), 10,000 x in DMSO 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 1000 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).

Optional Equipment

- Automated microfluidic electrophoresis station (Agilent Technologies 2100 Bioanalyzer).
- qPCR machine and library standards (for library quantification).
- Benchtop fluorometer and appropriate assays (for RNA quality control and library quantification).
- Agarose gels, dyes, and electrophoresis rig (for RNA quality control).

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.

The complete set of material, reagents, and labware necessary for RNA extraction and quality control is not listed. Consult Appendix A (p.22) for more information on RNA quality.

Consult Appendix E (p.29) 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. 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.
- 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 re-collected 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 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.
- 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 in steps 5 and 7 use a pipette set to 15 μl or 20 μl to ensure proper mixing.

Pipetting and Handling of (Viscous) Solutions

- Enzyme Mixes, **SS1**, **SS1x**, **PB**, and **PS** are viscous solutions which require care to pipette accurately. Quickly spin down the tubes to collect all liquid at the bottom of the tube. 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, 8, and 27 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 250.8 μl **FS2** ● (= 9.5 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 264 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.

5. Detailed Protocol

5.1 Library Generation

Preparation

First Strand cDNA Synthesis	RNA Removal	Second Strand Synthesis	Purification
FS1 – thawed at RT FS2 – thawed at RT FS1x – thawed at RT dT – thawed at RT FS2x – thawed at RT E1 – keep on ice or at -20 °C	RS1 – thawed at RT RS2 – thawed at RT	SS1 – thawed at 37 °C SS1x – thawed at 37 °C RSP – thawed at RT 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
Thermocycler 96-well PCR plate or 8-well strip PCR sealing films Plate centrifuge 85 °C, 3 min 42 °C / 50 °C, 15 min	Thermocycler 95 °C, 10 min cool down to 25 °C PCR sealing films Plate centrifuge	Thermocycler 98 °C, 1 min, then cool to 25 °C / 37 °C (0.5 °C/sec) 25 °C / 37 °C, 30 min 25 °C / 37 °C / 50 °C, 15 min PCR sealing films Plate centrifuge	96-well magnetic plate 96-well PCR plate

First Strand cDNA Synthesis - Reverse Transcription

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 015 reagent box (Cat. No. 015) with **FS1x** ● and **FS2x** ● from the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026). **FS1x** ● does not contain an RT 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.

1

Mix up to 5 µl of your RNA (typically 10 ng - 500 ng) with 5 µl **Custom Targeted Primers** (designed and provided by User, see also Appendix B, p.26) in a PCR plate or 8-well strip. If a smaller volume of RNA is used, add RNase-free water to a total volume of 10 µl. Mix well by pipetting. Seal the plate or PCR strips. Make sure the seal is closed tightly. Spin down the plate to make sure all liquid is collected at the bottom of the wells.

Denature the RNA / targeted primer mix for 3 minutes at 85 °C in a thermocycler and then cool down to 42 - 50 °C. **ATTENTION:** Skip this step for FFPE, degraded RNA samples (see Appendix D, p.28), and RNA inputs below 10 ng. **REMARK:** For targeted primers with a high T_m best practice would be to keep the RT reaction at an elevated temperature (up to 50 °C) at all times to prevent unspecific primer hybridization. Quickly spin down the plate to make sure all liquid is collected at the bottom of the wells before carefully removing the sealing foil.

2

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.

3

Add 10 µl of the **FS1x / FS2x / E1** mastermix for targeted priming, mix by pipetting, and seal the plate. Spin down the liquid at room temperature and incubate at 42 °C for 15 minutes. **REMARK:** For targeted primers with a high T_m RT reaction temperature can be increased up to 50 °C.

4

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

REMARK: The QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026) also contains a separate oligodT primer (**dT** ●) control containing an Illumina-compatible P7 (Read 2) linker sequence at its 5' end.

Mix up to 5 µl of your RNA (typically 10 - 500 ng) 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. If a smaller volume of RNA is used, add RNase-free water to a total volume of 10 µl. Mix well by pipetting. Seal the plate or PCR strips. Make sure the seal is closed tightly. Spin down the plate to make sure all liquid is collected at the bottom of the wells.

1

Denature the RNA / **FS1** mix for 3 minutes at 85 °C in a thermocycler and then cool down to 42 °C. Spin down the plate at room temperature to make sure all liquid is collected at the bottom of the wells before carefully removing the sealing foil. **ATTENTION:** Skip this step for FFPE, degraded RNA samples (see Appendix D, p.28), and RNA inputs below 10 ng.

2

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.

3

Add 5 µl of the **FS2 / E1** mastermix to each reaction, mix by pipetting, and seal the plate. Spin down the liquid at room temperature and incubate at 42 °C for 15 minutes.

4

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 hybridized to the RNA and reverse transcription is performed.

- 1 Mix up to 5 µl of your RNA (typically 10 - 500 ng) with 5 µl First Strand cDNA Synthesis Mix 1 (**FS1** ●) in a PCR plate or 8-well strip. If a smaller volume of RNA is used, add RNase-free water to a total volume of 10 µl. Mix well by pipetting. Seal the plate or PCR strips. Make sure the seal is closed tightly. Spin down the plate to make sure all liquid is collected at the bottom of the wells.

- 2 Denature the RNA / **FS1** mix for 3 minutes at 85 °C in a thermocycler and then cool down to 42 °C. Spin down the plate at room temperature to make sure all liquid is collected at the bottom of the wells before carefully removing the sealing foil. **ATTENTION:** Skip this step for FFPE, degraded RNA samples (see Appendix D, p.28), and RNA inputs below 10 ng.

- 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.

- 4 Add 10 µl of the **FS2 / E1** mastermix to each reaction, mix by pipetting, and seal the plate. Spin down the liquid at room temperature and incubate at 42 °C for 15 minutes.

RNA Removal

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

- 5 Add 5 µl RNA Removal Solution 1 (**RS1** ○) directly to the first strand cDNA synthesis reaction. Mix well and re-seal 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. **ATTENTION:** Reduce this step for RNA inputs below 1 ng total RNA to 5 minutes at 95 °C.

- 7 Add 5 µl of RNA Removal Solution 2 (**RS2** ○) and mix well. **REMARK:** Use a pipette set to 15 µl for efficient mixing.

Second Strand Synthesis

Target-specific Second Strand Synthesis (QuantSeq-Flex Second Strand Synthesis Module Cat. No. 028)

During this step the library is converted to dsDNA. Second strand synthesis is initiated by adding target-specific primers (designed and provided by User, see also Appendix B, p.26) containing an Illumina-compatible P5 (Read 1) linker sequence at their 5' end.

NOTE: At this point we recommend placing the Purification Beads (**PB**) for step 14 at room temperature to give them enough time to equilibrate.

ATTENTION: Second Strand Synthesis Mix 1 (**SS1x** ●) is a viscous solution and needs to be mixed thoroughly before use.

- 8 Prepare a Mastermix of 13 μl Second Strand Synthesis Mix 1 (**SS1x** ●) and 2 μl **Custom Targeted Primers** (designed and provided by User, see also Appendix B, p.26) per reaction. Mix well.

- 9 Add 15 μl of the **SS1x** / Primer mastermix per reaction. Mix well by pipetting, and seal the plate. **REMARK:** Use a pipette set to 40 μl for efficient mixing.

- 10 Incubate the plate for 1 minute at 98 °C in a thermocycler, and slowly cool down to 50 °C by setting the ramp speed to 10 % (0.5 °C/second). **REMARK:** Hybridization and reaction temperature depends on T_m of the primers but should be between 25 °C to 50 °C maximum. Quickly spin down the plate at room temperature before removing the sealing foil.

- 11 Prepare a mastermix containing 4 μl Second Strand Synthesis Mix 2 (**SS2** ●) and 1 μl Enzyme Mix 2 (**E2** ●). Mix well.

- 12 Add 5 μl of the **SS2** / **E2** mastermix per reaction. Mix well. **REMARK:** Use a pipette set to 40 μl for efficient mixing.

- 13 Incubate the reaction at 50 °C for 15 minutes. **ATTENTION:** Reaction temperature depends on the primers used but should not exceed 50 °C.

REMARK: The QuantSeq-Flex Second Strand Synthesis Module (Cat. No. 028) also contains a separate Random Second Strand Synthesis Primer (**RSP** ●) control containing an Illumina-compatible P5 (Read 1) linker sequence at its 5' end. If using this control, mix 13 μl Second Strand Synthesis Mix 1 (**SS1x** ●) and 2 μl Random Second Strand Synthesis Primer (**RSP** ●) and continue with step 9 as described in the Random Primed Second Strand Synthesis section on p.17.

Random primed Second Strand Synthesis (QuantSeq FWD kit components Cat. No. 015)

During this step the library is converted to dsDNA. Second strand synthesis is initiated by a random primer containing an Illumina-compatible linker sequence at its 5' end.

NOTE: At this point we recommend placing the Purification Beads (**PB**) for step 14 at room temperature to give them enough time to equilibrate.

ATTENTION: Second Strand Synthesis Mix 1 (**SS1** ●) is a viscous solution and needs to be mixed thoroughly before use.

- 8 Add 15 µl Second Strand Synthesis Mix 1 (**SS1** ●). Mix well by pipetting, and seal the plate. **OPTIONAL:** If longer insert sizes are desired use only 7.5 µl **SS1** ● and add 7.5 µl RNase-free water. Please keep in mind that cycle numbers may need to be increased if **SS1** ● is diluted (+3 cycles). We recommend taking advantage of the qPCR assay as described in Appendix A, p.22. **REMARK:** Use a pipette set to 40 µl for efficient mixing.
- 9 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).
- 10 Incubate the reaction for 30 minutes at 25 °C. Quickly spin down the plate at room temperature before removing the sealing foil.
- 11 Prepare a mastermix containing 4 µl Second Strand Synthesis Mix 2 (**SS2** ●) and 1 µl Enzyme Mix 2 (**E2** ●). Mix well.
- 12 Add 5 µl of the **SS2** / **E2** mastermix per reaction. Mix well. **REMARK:** Use a pipette set to 40 µl for efficient mixing.
- 13 Incubate the reaction at 25 °C for 15 minutes. **REMARK:** For targeted primers with a high T_m reaction temperature can be increased up to 50 °C.

NOTE: **SS1** ● dilution to increase insert sizes is only applicable if random priming is used during second strand synthesis. For targeted primers insert sizes are determined by the primer binding site hence **SS1x** ● dilutions in combination with targeted priming has no effect on the insert size.

Purification

The double-stranded library is purified by using magnetic beads to remove all reaction components. The Purification Beads (**PB**) 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.

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

15 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).

16 Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

17 Remove the plate from the magnet and add 40 μ l of Elution Buffer (**EB**). Resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.

18 Add 72 μ l of Purification Solution (**PS**) to the beads / **EB** mix to re-precipitate the library. Mix thoroughly, and incubate for 5 minutes at room temperature. **ATTENTION:** For low input RNA, FFPE, or degraded RNA add only 48 μ l (**PS**) (see Appendix B, p.26 and Appendix D, p.28).

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

20 Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

21 Add 120 μ l of 80 % EtOH, and wash 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.

22 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.

23 Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Do not let the beads dry too long (visible cracks appear) as this will negatively influence the elution and the resulting library yield.

24 Add 20 μ l 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.

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

26 Transfer 17 μ l of the clear supernatant into a fresh PCR plate. Make sure not to transfer any beads. Libraries can be stored at -20 °C for later amplification. See Appendix A, p.22 for qPCR options.

5.2 Library Amplification

Preparation

PCR	Purification
PCR – thawed at RT E3 – keep on ice or at -20 °C BC – thawed at RT; spin down before opening!	PB – stored at +4 °C PS – stored at +4 °C 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 72 °C, 1 min 10 °C, ∞ <small>best determined by qPCR see p.22</small>	96-well magnetic plate 96-well PCR plate PCR sealing films Plate centrifuge

PCR

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

ATTENTION: Cycle numbers differ depending on the RNA and the target primers used.

Lexogen offers a PCR Add-on Kit for Illumina (Cat. No. 020.96) which is highly recommended to be used for qPCR determination of the appropriate endpoint PCR cycle number on diluted cDNA samples. For details see Appendix A, p.22.

NOTE: At this point we recommend placing the Purification Beads (**PB**) for step 31 at room temperature to give them enough time to equilibrate.

27 Prepare a mastermix containing 7 µl of PCR Mix (**PCR** ●) and 1 µl Enzyme Mix 3 (**E3** ●) per reaction.

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

Add 5 µl of the respective external Barcode Primer (**BC01-96**, in 96-well plate, **ATTENTION:** Spin down before opening! Avoid cross contamination!). Mix well by pipetting. Seal the plate and quickly spin down to make sure all liquid is collected at the bottom of the well. **RECOMMENDED:** For targeted approaches, we recommend to perform a qPCR and use 5 µl of Barcode 00 (**BC00** ●) included in the PCR Add-on Kit for Illumina Cat. No. 020.96) at this step for the qPCR and the respective Barcode primer (**BC01-96**, in 96-well plate) for the subsequent endpoint PCR (see Appendix A, p.22).

30 Conduct x cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, x 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. .

Purification

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

- 31 Add 30 μ l of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature. **ATTENTION:** For low input RNA, FFPE, or degraded RNA add only 27 μ l Purification Beads (**PB**) (see Appendix D, p.28).

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

- 33 Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

- 34 Add 30 μ l of Elution Buffer (**EB**), remove the plate from the magnet and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.

- 35 Add 30 μ l of Purification Solution (**PS**) to the beads / **EB** mix to re-precipitate the library. Mix thoroughly, and incubate for 5 minutes at room temperature.

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

- 37 Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

- 38 Add 120 μ l of 80 % EtOH, and wash 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.

- 39 Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.

- 40 Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Do not let the beads dry too long (visible cracks appear) as this will negatively influence the elution and hence the resulting library yield.

- 41 Add 20 μ l 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.

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

- 43 Transfer 15 - 17 μ l of the supernatant into a fresh PCR plate. Make sure not to transfer any beads.

- 44 At this point, the libraries are finished and ready for quality control (Appendix E, p.29), pooling (for multiplexing; see Appendix F, p.31), and cluster generation.

6. Short Procedure - Targeted Priming

ATTENTION: Spin down solutions before opening tubes or plates!

200 min

Library Generation

<input type="checkbox"/>	Mix 5 µl RNA and 5 µl Custom Targeted Primers .	Targeted First Strand cDNA Synthesis
<input type="checkbox"/>	Incubate at 85 °C for 3 min, cool to 42 - 50 °C. Skip this step for low input, FFPE, or low quality RNA.	
<input type="checkbox"/>	For targeted priming prepare a mastermix with 5 µl FS1x ●, 4.5 µl FS2x ● and 0.5 µl E1 ● per reaction.	
<input type="checkbox"/>	Add 10 µl FS1x / FS2x / E1 mix for targeted priming per reaction.	
<input type="checkbox"/>	Incubate for 15 min at 42 °C (or up to 50 °C for targeted primers with a high T _m).	
<input type="checkbox"/>	Add 5 µl RS1 ○, mix well.	RNA Removal
<input type="checkbox"/>	Incubate 10 min at 95 °C, then cool to 25 °C. Reduce to 5 min 95 °C for < 1 ng RNA input.	
<input type="checkbox"/>	Add 5 µl RS2 ○, mix well.	
<input type="checkbox"/>	Add 13 µl SS1x ● and 2 µl Custom Targeted Primers per reaction, mix well.	Targeted Second Strand cDNA Synthesis
<input type="checkbox"/>	Incubate 1 min at 98 °C, slowly ramp down (0.5 °C/sec) to 50 °C (targeted primers).	
<input type="checkbox"/>	Prepare a mastermix with 4 µl SS2 ● and 1 µl E2 ● per reaction.	
<input type="checkbox"/>	Add 5 µl SS2 / E2 mix per reaction, mix well.	
<input type="checkbox"/>	Incubate 15 min at 50 °C. ATTENTION: Reaction temperature depends on the targeted primers used but should not exceed 50 °C!	
<input type="checkbox"/>	Add 20 µl PB per reaction, mix well, incubate 5 min.	Purification
<input type="checkbox"/>	Place on magnet for 2 - 5 min, remove and discard supernatant.	
<input type="checkbox"/>	Add 40 µl EB , mix well, incubate 2 min at RT.	
<input type="checkbox"/>	Add 72 µl PS (or 48 µl PS for low input, FFPE, or low quality RNA), mix well, incubate 5 min at RT.	
<input type="checkbox"/>	Place on magnet for 2 - 5 min, remove and discard supernatant.	
<input type="checkbox"/>	Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.	
<input type="checkbox"/>	Dry beads for 5 - 10 min. ATTENTION: Do not let the beads dry too long!	
<input type="checkbox"/>	Add 20 µl EB , mix well, incubate 2 min at RT.	
<input type="checkbox"/>	Place on magnet for 2 - 5 min, transfer 17 µl of the supernatant into a fresh PCR plate.	

70 min

Library Amplification

<input type="checkbox"/>	Prepare a mastermix with 7 µl PCR ● and 1 µl E3 ● per reaction, mix well.	PCR						
<input type="checkbox"/>	Add 8 µl PCR / E3 premix to 17 µl of each purified library.							
<input type="checkbox"/>	Add 5 µl BC (from the 96-well plate) for each reaction, mix well.							
<input type="checkbox"/>	PCR: 98 °C, 30 sec							
	<table border="0"> <tr> <td>98 °C, 10 sec</td> <td rowspan="4">} x use qPCR to determine cycle number; see p.22</td> </tr> <tr> <td>65 °C, 20 sec</td> </tr> <tr> <td>72 °C, 30 sec</td> </tr> <tr> <td>72 °C, 1 min</td> </tr> <tr> <td>10 °C, ∞</td> <td></td> </tr> </table>		98 °C, 10 sec	} x use qPCR to determine cycle number; see p.22	65 °C, 20 sec	72 °C, 30 sec	72 °C, 1 min	10 °C, ∞
98 °C, 10 sec	} x use qPCR to determine cycle number; see p.22							
65 °C, 20 sec								
72 °C, 30 sec								
72 °C, 1 min								
10 °C, ∞								
<input type="checkbox"/>	Add 30 µl PB (or 27 µl PB for low quality/low input/FFPE RNA) per reaction, mix well, incubate 5 min.	Purification						
<input type="checkbox"/>	Place on magnet for 5 min, remove and discard supernatant.							
<input type="checkbox"/>	Add 30 µl EB , mix well, incubate 2 min at RT.							
<input type="checkbox"/>	Add 30 µl PS , mix well, incubate 5 min at RT.							
<input type="checkbox"/>	Place on magnet for 2 - 5 min, remove and discard supernatant.							
<input type="checkbox"/>	Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.							
<input type="checkbox"/>	Air dry beads for 5 - 10 minutes. ATTENTION: Do not let the beads dry too long!							
<input type="checkbox"/>	Add 20 µl EB , mix well, incubate 2 min at RT.							
<input type="checkbox"/>	Place on magnet for 2 - 5 min, transfer 15 - 17 µl of the supernatant into a fresh PCR plate.							

7. Appendix A: RNA Requirements

RNA Amount and 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. By using targeted priming only for one of the cDNA synthesis steps e.g., targeted primers only using 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. However, take into consideration that your targeted second strand synthesis primers should be located near the 3' end (optimal within 100 - 500 bases upstream of the poly A tail).

The amount of total RNA required for QuantSeq depends on the poly(A) RNA content or target RNA of the sample in question. QuantSeq with oligodT primed first strand synthesis and random primed second strand synthesis was extensively tested with various cell cultures, mouse and plant tissues, yeast, fungi, and human reference RNA (Universal Human Reference RNA (UHRR) and Brain Reference RNA). Typical inputs of 500 ng total RNA generate high quality QuantSeq 3' mRNA-Seq libraries (oligodT primed RT and random primed second strand synthesis) for single-end 50 nt (SR50) or 100 nt sequencing (SR100) with 12 cycles of library amplification. For mRNA-rich tissues (such as kidney, liver, and brain) input RNA may be decreased to 50 ng without adjusting the protocol. Lower RNA inputs (500 pg - 10 ng) or low quality RNA input may require protocol adjustments (see Appendix D, p.28).

QuantSeq-Flex is a flexible, open kit allowing the usage of custom primers for targeted sequencing. As targeted sequencing depends on the tissue and target primers used, optimal cycle numbers need to be determined by qPCR. Protocol adjustments such as increased reaction temperatures may be beneficial for increasing the specificity of the reaction when using custom primers.

With reduced total RNA input and / or custom primers cycle numbers need to be adjusted for which we recommend using a qPCR assay (for details see p.23).

The input requirements for your particular experiment may be different, depending on the primers used and the abundance of the target RNAs in your tissue. Overcycling of libraries - indicated by a second high molecular weight peak between 1000 - 9000 bp in a Bioanalyzer trace - should be prevented as this may lead to distortions in transcript abundance and library quantification.

As a starting point, we recommend performing the protocol initially with 500 ng total RNA and performing a qPCR assay to determine exact cycle numbers for your endpoint PCR if custom primers were used.

Lexogen offers a PCR Add-on Kit for Illumina (Cat. No. 020.96) for this purpose.

qPCR to Determine the Exact Cycle Number of Your Endpoint PCRs

For determining the cycle number of your endpoint PCR, please use 5 µl of Barcode 00 (**BC00** ●) included in the PCR Add-on Kit for Illumina (Cat. No. 020.96) in step 29 of the protocol. Dilute the double stranded library from step 26 to 34 µl by adding 17 µl Elution Buffer (**EB**) (i.e., 1 : 2 dilution) in order to have enough template for qPCR and endpoint PCR. Add 17 µl of the diluted cDNA into a PCR reaction. To render this PCR reaction quantifiable (qPCR), simply add SYBR Green I (or an equivalent fluorophore) in a final concentration of 0.1x. For 0.1x SYBR Green I add 1.2 µl 2.5x SYBR Green I solution (1:4,000 SYBR Green I dilution, diluted in DMSO). The total PCR reaction volume will be 31.2 µl. Alternatively, if 8 qPCRs are run at the same time, best practice would be to prepare a mastermix with 0.15 µl of a 20x SYBR Green I solution per reaction. SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines has to be adjusted manually. Overcycle this initial qPCR (40 cycles or even 50 if little input material was used or low abundant transcripts were targeted; include a no template control!), and then determine **the maximum fluorescence value** at which the fluorescence reaches a plateau. Calculate where the fluorescence is 33 % of the maximum, and use the corresponding cycle number for the endpoint PCR with the remaining 17 µl of the template. There is no need to purify or analyze the overcycled PCR reaction on a Bioanalyzer.

For similar samples that have not been diluted for the qPCR assay the cycle number determined in the overcycled qPCR has to be reduced by 1 cycle.

EXAMPLE: 500 ng input RNA was used for generating two libraries. The cDNA of library 1 was diluted and half of the template was inserted into the qPCR assay. The cycle number determined in the overcycled qPCR (33 % of the maximum fluorescence) was 13 cycles. The remaining half of the template should be amplified with 13 cycles, whereas the undiluted cDNA of library 2 can be amplified with 12 cycles, as here double the amount of template is inserted into the endpoint PCR.

Reamplification of Barcoded Libraries with the PCR Add-On Kit

Lexogen's PCR Add-on Kit also contains a Reamplification Primer (**RE** ○) that can be used to reamplify already barcoded libraries if they were undercycled to get enough material for sequencing. For details please refer to the PCR Add-on Kit (Cat. No. 020.96) Instruction Manual.

ATTENTION: Do not use the **BC00** ● for the reamplification of already barcoded libraries! This will lead to a loss of barcodes and to a mixed and not assignable sequence pool in an NGS run.

ATTENTION: Do not use the Reamplification Primer (**RE** ○) for a qPCR assay on the cDNA-library as the cDNA lacks binding sites for the Reamplification Primer. **RE** ○ can be only used on already amplified PCR libraries.

RNA Integrity

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). As QuantSeq specifically targets the 3' end of transcripts even RNAs with a lower RIN are suitable as input material.

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, such as QuantSeq, as only one fragment will be generated for each transcript. Optional an rRNA depletion method, which also removed mt-rRNAs, such as Lexogen's RiboCop rRNA Depletion Kit (Cat. No. 037.08, 037.24, and 037.96) can be used before starting the QuantSeq library preparation, if it is essential to remove mt-rRNA transcripts.

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 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 be sheared.

SIRVs Spike-in RNA Variant Control Mixes

Lexogen offers a set of artificial spike in transcripts called SIRVs (Spike-In RNA Variant Control Mixes, Cat. No. 025.03), to serve as a control and anchor set for the comparison of RNA-Seq experiments. The SIRV sequences enable bioinformatic algorithms to accurately map, assemble, and quantify isoforms, and provide the means for the validation of these algorithms in conjunction with the preceding RNA-Seq pipelines. The SIRVs consist of 69 artificial RNA transcripts with no identity to any known genomic sequences hence they can be spiked into any RNA. Each SIRVs box contains 3 tubes labeled E0, E1, or E2. Each mix contains all 69 SIRV transcripts, but at different molar concentrations to each other. SIRV Mixes can be used as single spike-ins, or as a combination of two or three different SIRV mixes for the assessment of differential gene expression.

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.). These sets of RNAs 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 over-cycling 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.

8. Appendix B: 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 14 and 18). The full Illumina P7 (Read 2) adapter sequence will only be introduced during PCR (step 29).

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

5' GTTCAGACGTGTGCTCTTCCGATCT- Target sequence 3'

Here the target sequence has to be the reverse complement of the RNA-sequence in question. The chosen target sequence should be as specific as possible with a T_m that is as close as possible to the intended reaction temperature. In most cases 20 nt are enough. Target 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 (Illumina-sequence + targeted sequence). Optionally, a 6 - 8 nt long molecular index between adapter sequence and target sequence could also be included, but then a paired-end sequencing run is required for read out.

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 14 and 18). The full Illumina P5 (Read 1) adapter sequence will only be introduced during PCR (step 29). **REMARK:** Introduction of 1 - 3 phosphorothioate linkages (PTOs) at the 3' end of the second strand synthesis primer may increase specificity.

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.

We recommend introducing a 6 - 8 nt long molecular index 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 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.

9. Appendix C: Primer Concentrations

First Strand Synthesis

The concentration of a target-specific RT primer should be around 12.5 nM - 1.25 μ M final concentration (in this case it would mean you would need 5 μ l of a 50 nM - 5 μ M target-specific primer). The total concentration of oligos in the first strand synthesis reaction should not exceed 2 μ M. The higher the primer concentration the higher the likelihood of unspecific binding.

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

For determination of the optimal number of PCR cycles we strongly recommend taking advantage of the qPCR assay as described in Appendix A, p.22.

Second Strand Synthesis

The concentration of a target-specific second strand synthesis primer should be around 10 - 100 nM final concentration (in this case it would mean you would need 2 μ l of a 250 nM - 2.5 μ M target-specific primer). The total concentration of all oligos in the second strand synthesis reaction should not exceed 2 μ M. The higher the primer concentration the higher the likelihood of unspecific binding.

However, the exact primer concentration and reaction temperature (25 °C, 37 °C, 42 °C, 50 °C) strongly depends on the custom primer(s) used and has to be optimized accordingly.

For determination of the optimal number of PCR cycles we strongly recommend taking advantage of the qPCR assay as described in Appendix A, p.22.

10. Appendix D: 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 50 bp - 250 bp) in order to account for the degraded RNA starting material. For FFPE samples only minor protocol adjustments are required, such as skipping step 2 and reducing the addition of **PS** in step 18 to 48 μ l as well as using only 27 μ l PB in step 31.

In addition to an RNA Integrity Number (RIN), the quality of FFPE RNA is assessed by the DV200 value. The DV200 value is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV200, 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 using 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. However, take into consideration that your targeted Second Strand Synthesis Primers should be located near the 3' end (optimal within 100 -500 bases upstream of the poly A tail).

To determine the exact cycle number of your endpoint PCR, we would strongly recommend using the PCR Add-On Kit (Cat. No. 020.96) and **taking advantage of the qPCR assay** as described on p.22.

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 μ l lane mix plus 45 μ l **PB**), incubating 5 minutes at room temperature, and following the protocol from step 32 on again may be necessary.

11. Appendix E: 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 μ l of a QuantSeq library produced according to the directions in this manual is sufficient for analysis. Depending on the minimum sample loading requirements for each instrument, 1 μ l of the finished library may be diluted to the required volume (e.g., 2 μ l sample for TapeStation and 10 μ l for LabChip GX II).

More accurate library quantification can be achieved with custom or commercially available qPCR assays. With these assays, the relative or absolute abundance of amplifiable fragments contained in a finished QuantSeq library is calculated by comparing 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. Most UV-Vis spectrophotometers are not sensitive enough at these concentrations to accurately quantify NGS libraries and should be avoided.

Typical Results

QuantSeq-Flex libraries are intended for a high degree of multiplexing, and 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. 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 used.

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 libraries. 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) input RNA, respectively.

A shorter side-product caused by priming of the second strand synthesis oligo on the oligodT 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.

A second peak in high molecular weight regions (between 1000 - 9000 bp) is an indication of overcycling. This could occur if cycle numbers are increased too much to compensate for lower input material.

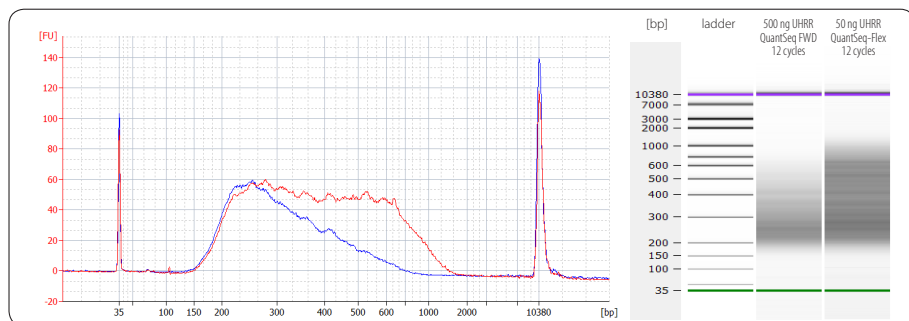


Figure 3. Bioanalyzer traces of QuantSeq-Flex (red trace) with oligodT primed first strand synthesis and random primed second strand synthesis vs QuantSeq FWD libraries (blue trace) synthesized from 500 ng total RNA input, amplified with 12 cycles. Input RNA was Universal Human Reference RNA (UHRR).

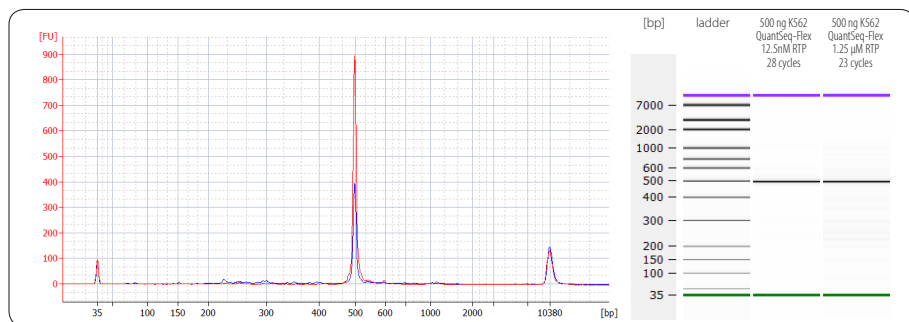


Figure 4. Bioanalyzer traces of QuantSeq-Flex libraries targeting for BCR-ABL fusion transcripts. 500 ng K562 total RNA was used as input material. 12.5 nM final concentration of a targeted first strand synthesis primer (5' CGTGTCTCTCCGATCTGTGTGACTGGCGTGATGTAGTGTGCTGG 3') (red trace) was amplified for 28 cycles. Increasing the first strand synthesis primer concentration by a factor of 100 (1.25 μM final concentration) reduced the required cycle number to 23 cycles (blue trace), but also results in minor side products. 100 nM final concentration of the second strand synthesis primer (5' CACGACGCTCTCCGATCTACAGATTCCGCTGACCATCAATAAG 3') was used for all three libraries. First and second strand synthesis were performed at 50 °C.

12. Appendix F: Multiplexing

QuantSeq libraries are designed for a high degree of multiplexing. External barcodes are introduced during the PCR amplification step.

External Barcodes

External Barcodes allowing up to 96 samples to be sequenced per lane on an Illumina flow cell are included in the kit in the Barcode Plate (**BC**). External Barcodes are 6 nt long and require an additional index-specific sequencing reaction.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC01: ACATTA	BC02: GGTGAG	BC03: CGAAGG	BC04: AAGACA	BC05: TAATCG	BC06: CGCAAC	BC07: AATAGC	BC08: TTAACT	BC09: AATGAA	BC10: GATTGT	BC11: ATAAGA	BC12: GCCACA
B	BC13: GAACCT	BC14: CGGTTA	BC15: AACGCC	BC16: CAGATG	BC17: GATCAC	BC18: CGCGGA	BC19: CCTAAG	BC20: GGCTGC	BC21: ACCAGT	BC22: GTGCCA	BC23: AGATAG	BC24: TCGAGG
C	BC25: ACAACG	BC26: GCGCTG	BC27: CAAGCA	BC28: GTTACC	BC29: CTCTCG	BC30: CCAATT	BC31: TTCGAG	BC32: CGTCGC	BC33: TGTGCA	BC34: ACCGTG	BC35: ATACTG	BC36: ATGAAC
D	BC37: AGTTGA	BC38: GACGAT	BC39: CACACT	BC40: CAGCGT	BC41: TGCTAT	BC42: TCTTAA	BC43: CCGCAA	BC44: CTCCAT	BC45: GTCAGG	BC46: ACGTCT	BC47: GAGTCC	BC48: GACATC
E	BC49: AGGCAT	BC50: ACCTAC	BC51: TGGATT	BC52: GCAGCC	BC53: CGCCTG	BC54: CCGACC	BC55: TATGTC	BC56: TGACAC	BC57: ACAGAT	BC58: AGACCA	BC59: GCTCGA	BC60: ATGGCG
F	BC61: GAAGTG	BC62: AGAATC	BC63: GCGAAT	BC64: CGATCT	BC65: CATCTA	BC66: AAGTGG	BC67: TGCACG	BC68: TCGTTC	BC69: ACACGC	BC70: GTAGAA	BC71: AGTACT	BC72: GCATGG
G	BC73: AACAAAG	BC74: AACCGA	BC75: TGGCGA	BC76: CACTAA	BC77: AAGCTC	BC78: TACCTT	BC79: CTAGTC	BC80: AATCCG	BC81: GTGTAG	BC82: ACTCTT	BC83: TCAGGA	BC84: ATTGGT
H	BC85: TTGGTA	BC86: CAACAG	BC87: CAATGC	BC88: GGAGGT	BC89: CAGGAC	BC90: GGCCAA	BC91: CTCATA	BC92: CCTGCT	BC93: GGTATA	BC94: TTCCGC	BC95: TAGGCT	BC96: ATATCC

External barcode sequences are available for download at www.lexogen.com.

QuantSeq is specifically designed for multiplexing 48 or 96 samples per sequencing lane (depending on the intended read depth).

The 24 reaction QuantSeq kits (Cat. No. 015.24, Cat. No. 016.24) include Barcode Set 1 (BC01/BC13/BC25/BC37/BC49/BC61/BC73, and BC85), Barcode Set 2 (BC02/BC14/BC26/BC38/BC50/BC62/BC74, and BC86), and Barcode Set 3 (BC03/BC15/BC27/BC39/BC51/BC63/BC75, and BC87). The 96 reaction kits (Cat. No. 015.96, Cat. No. 015.2x96, Cat. No. 016.96, Cat. No. 016.2x96) includes all Barcode Sets (Set 1 - 12) and here Barcodes can be combined across rows (Set A: BC01 - 12, Set B: BC13 - 24, and so on) or columns (Set 1 - 12).

In general, we recommend processing a minimum of 8 samples, better 12 at a time and using a complete set of 8 or 12 Barcodes for multiplexing (e.g., Set 1 or Set A if a 96 reaction kit is used, respectively). However, if fewer Barcodes are required care should be taken to always use sets of Barcodes which give a signal in both lasers (red and green channels) for each nucleotide position. Sets 1 - 12 and A - H fulfill these criteria. The individual libraries within a lane should be mixed at an equimolar ratio to ensure this balance.

13. Appendix G: Sequencing*

General

The amount of library loaded onto the flowcell will greatly influence the number of clusters generated. Each sequencing facility has slightly different preferences of how much to load. From our experience a good starting point is to load between 7 and 14 pM (pmol/l) of a QuantSeq library onto the flowcell.

A schematic representation of the QuantSeq FWD and QuantSeq-Flex libraries (Cat. No. 015 and Cat. No. 26, 28, 33, 34, 35) is shown below. The required sequencing primers are also listed. If a molecular barcode (see Appendix B, p.26) was introduced for QuantSeq-Flex Targeted second strand synthesis a N sequence corresponding to the molecular barcode is found at the beginning of Read 1 (i.e., between Read 1 sequencing primer and target sequence).

QuantSeq FWD and QuantSeq-Flex Libraries with External Barcodes

External Barcodes (6 nt) are introduced during PCR (step 29).

For QuantSeq FWD and QuantSeq-Flex libraries, Read 1 directly corresponds to the RNA sequence.

```
5'-(Read 1 Sequencing Primer)-3'
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTCCGATCT- (Insert...
3' TTAGCATGCCCGTGGTGGCTCTAGATGTGAGAAAGGATGTGCTGCGAGAAGGCTAGA- (Insert...

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

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

5' ACACTCTTTCCCTACACGACGCTCTCCGATCT 3'

Index Read: Multiplexing Index Read Sequencing Primer (not supplied):

5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

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

5' GTGACTGGAGTTCAGACGTGTGCTCTCCGATCT 3'

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.

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

14. Appendix H: 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 contains the Read 1 linker sequence in the 5' part of the second strand synthesis primer, hence NGS reads are generated towards the poly(A) tail.

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.

De-Multiplexing

External Barcodes: The barcode is contained in the Index Read, and demultiplexing can be carried out by the standard Illumina pipeline.

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 (Cat.No. 015) and QuantSeq-Flex data.

Annotation

Please visit our website (www.lexogen.com) for an up-to-date table of suggested species-specific annotations and comments.

15. Appendix I: Revision History

Revision date/ Publication No.	Change	Page
015UG058V0110	Format changes.	
	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 seq run for read-out).	25
015UG058V0102	SSS at temperatures of up to 50 °C.	15, 20, 25
	Remark to includes PTOs in SSS oligos for increased specificity	24
	Updated recommendation for primer concentrations.	24
	Example for targeted sequencing (BCR-ABL fusion transcript).	28
015UG058V0101	Minor changes	
015UG058V0100	Initial Release	

16. Notes

The background of the page is white with a decorative pattern of translucent blue spheres of various sizes. Some spheres are connected by thin, light blue lines, creating a network-like structure. The spheres have a glossy, 3D effect with highlights and shadows.

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

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