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3′ mRNA-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)
025 (SIRVs Spike-in RNA Variant Control Mixes)
026 (QuantSeq-Flex First Strand Synthesis Module)
028 (QuantSeq-Flex Second Strand Synthesis Module)
033 (QuantSeq-Flex Targeted mRNA-Seq Library Prep Kit with First Strand Synthesis Module)
034 (QuantSeq-Flex Targeted mRNA-Seq Library Prep Kit with Second Strand Synthesis Module)
035 (QuantSeq-Flex Targeted mRNA-Seq Library Prep Kit with First and Second Strand Synthesis Modules)
037 (RiboCop rRNA Depletion Kit)

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1. Overview

Lexogen's QuantSeq kit provides a library preparation protocol designed to generate Illumina-compatible libraries from polyadenylated RNA within 4.5 hours. The QuantSeq protocol generates only one fragment per transcript, resulting in extremely accurate gene expression values, and the sequences obtained are close to the 3' end of the transcripts.
QuantSeq is available with two read directions: QuantSeq Forward (FWD, Cat. No. 015) contains the Read 1 linker sequence in the second strand synthesis primer, hence NGS reads are generated towards the poly(A) tail and directly correspond to the mRNA sequence. To pinpoint the exact 3' end longer reads may be required. Although paired-end sequencing is possible, we do not recommend it for Cat. No. 015. Read 2 would start with the poly(T) stretch, and as a result of sequencing through the homopolymer stretch the quality of Read 2 would be very low.
For QuantSeq Reverse (REV, Cat. No. 016) the Read 1 linker sequence is introduced by the oligodT primer. Here, a Custom Sequencing Primer (**CSP ●** Version 2, included in the kit) is required for Read 1. The sequence generated during Read 1 corresponds to the cDNA. If paired-end sequencing is desired, we strongly recommend using QuantSeq REV (Cat. No. 016) and the **CSP ●** Version 2 for Read 1. With QuantSeq REV and the **CSP ●** it is possible to exactly pinpoint the 3' end in Read 1.
Both QuantSeq FWD and QuantSeq REV maintain strand-specificity and allow 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. Information regarding input RNA requirements can be found in Appendix A (p.17).

Library generation is initiated by oligodT priming. The primer already contains Illumina-compatible linker sequences. After first strand synthesis the RNA is removed and second strand synthesis is initiated by random priming and a DNA polymerase. The random primer also contains Illumina-compatible linker sequences. No purification is required between first and second strand synthesis. The insert size is optimized for shorter reads (SR50, PE50, SR100, PE100).

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 F, p.26 for a schematic representation of the finished library). External barcodes are included in the QuantSeq kit and are introduced during the PCR amplification step (Appendix E, p.26).
Library quantification can be performed with standard protocols and is further discussed in Appendix D (p.23).
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 G (p.29).

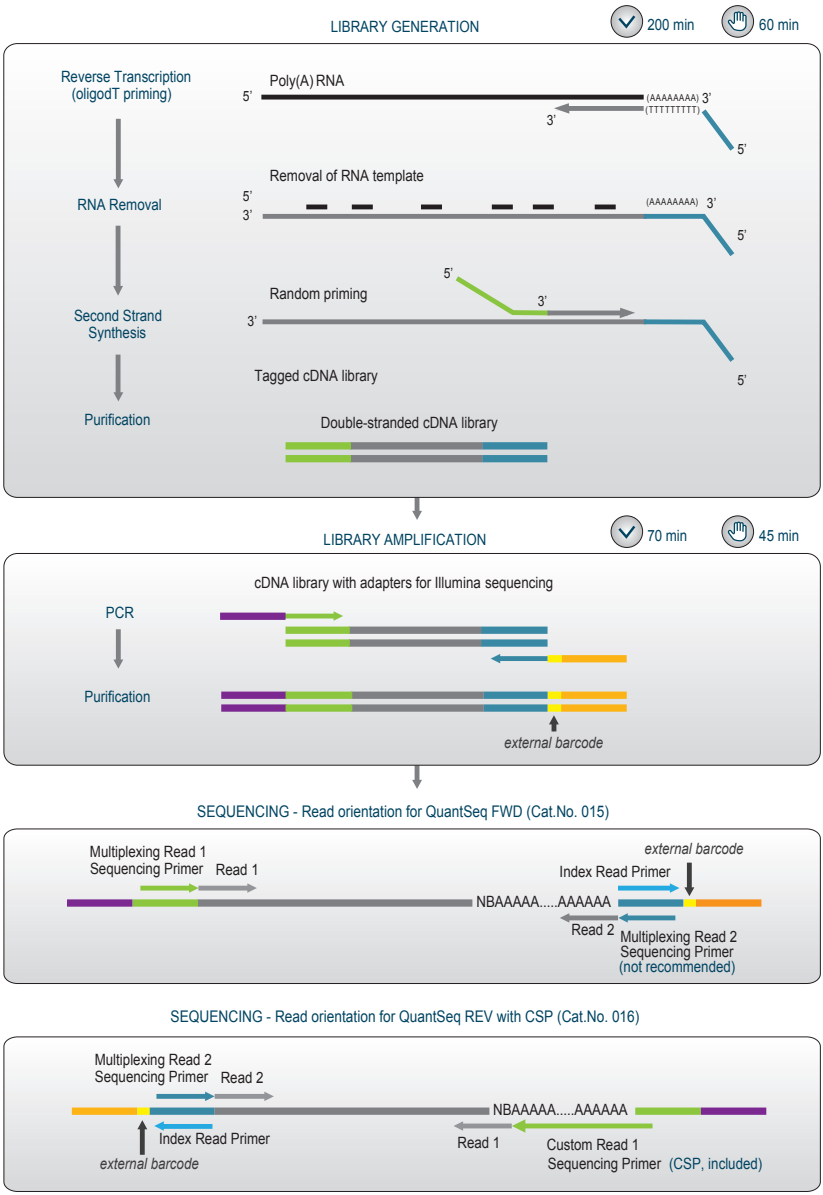

Figure 1. Schematic overview of the QuantSeq FWD library preparation workflow (Cat. No. 015). For QuantSeq REV (Cat. No. 016) the position of adapters for Read 1 (green) and Read 2 (blue) are switched. Sequencing Read orientation for QuantSeq FWD and QuantSeq REV is depicted as well. For QuantSeq FWD, Read 1 reflects the mRNA sequence. Paired-end Sequencing is not recommended for QuantSeq FWD. QuantSeq REV is suitable for paired-end sequencing, and Read 1 reflects the cDNA sequence. A Custom Sequencing Primer (CSP Version 2, included in the kit) is required for Read 1.

2. Kit Components and Storage Conditions

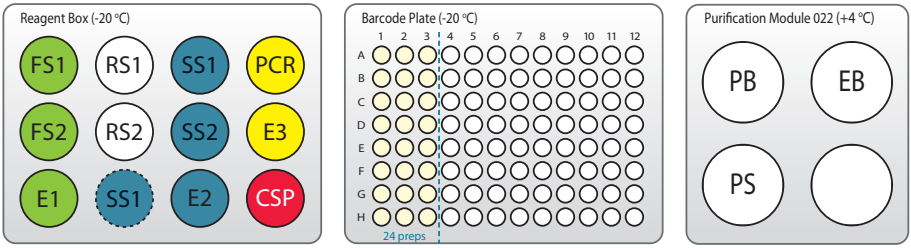

Figure 2. Location of kit components. For the 24 prep kit the dotted SS1 tube is missing and the barcode plate is only filled with set 1-3 (up to the blue dotted line). CSP (red lid color) is only required and included for QuantSeq REV (Cat. No. 016.24, Cat. No. 016.96, Cat. No. 016.2x96). All kits include Purification Modules.

Kit Component	Tube Label	Volume* provided for		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** ●	250.8 µl	1003.2 µl	-20 °C
Enzyme Mix 1	**E1** ●	13.2 µl	52.8 µl	-20 °C
RNA Removal Solution 1	**RS1** ○	132 µl	528 µl	-20 °C
RNA Removal Solution 2	**RS2** ○	132 µl	528 µl	-20 °C
Second Strand Synthesis Mix 1	**SS1** ●	396 µl	1584 µl	-20 °C
Second Strand Synthesis Mix 2	**SS2** ●	105.6 µl	422.4 µl	-20 °C
Enzyme Mix 2	**E2** ●	26.4 µl	105.6 µl	-20 °C
PCR Mix	**PCR** ●	184.8µl	739.2 µl	-20 °C
Enzyme Mix 3	**E3** ●	26.4 µl	105.6 µl	-20 °C
Barcode Plate (96-well plate)	**BC**	5 µl / reaction		-20 °C
Custom Sequencing Primer Version 2 (100 µM)**	**CSP** ●	25 µl	50 µl	-20 °C
Purification Module (Cat. No. 022) included in the kit				
Purification Beads	**PB**	1320 µl	5280 µl	+4 °C
Purification Solution	**PS**	2693 µl	10771 µl	+4 °C
Elution Buffer	**EB**	2904 µl	11616 µl	-20 °C/+4 °C

** only required for QuantSeq REV 016 *including a 10 % surplus

Upon receiving the QuantSeq kit, store the bottles **PB**, **PS**, and **EB** at +4 °C and the rest of the kit in a -20 °C freezer. **EB** can be stored either at +4 °C or -20 °C. Before use, check the contents of **PS**. If a precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

ATTENTION: The Custom Sequencing Primer (**CSP** ●) is only included and required for QuantSeq REV (Cat. No. 016) libraries. **CSP** ● has to be provided to the sequencing facility together with the lane mix. For further details on the usage of the CSP and the required volumes please consult Appendix F, p.26. **Forward this information to your sequencing facility before starting a sequencing run.**

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 (for 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 low binding 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.17) for more information on RNA quality.

Consult Appendix D (p.23) 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 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 just 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 ra-

tes, 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. Preheat lid to 105 °C, in case this has to be adjusted manually.

- 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** ●, **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, 10, and 26 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 **E1** – keep on ice or at -20 °C	**RS1** – thawed at RT **RS2** – thawed at RT	**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
Thermocycler 96-well PCR plate or 8-well strip PCR sealing films Plate centrifuge 85 °C, 3 min 42 °C, 15 min	Thermocycler 95 °C, 10 min cool down to 25 °C PCR sealing films Plate centrifuge	Thermocycler 98 °C, 1 min cool to 25 °C (0.5 °C/sec) 25 °C, 30 min 25 °C, 15 min PCR sealing films Plate centrifuge	96-well magnetic plate 96-well PCR plate

First Strand cDNA Synthesis - Reverse Transcription

An oligodT primer containing an Illumina-compatible sequence at its 5'end is hybridized to the RNA and reverse transcription is performed. QuantSeq libraries are intended for a high degree of multiplexing, so we recommend processing a minimum of 8 libraries at a time.

1. Mix up to 5 µl of your RNA (typically 500 ng, see also Appendix B, p.21 and Appendix C, p.22) 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. **REMARK:** For longer insert sizes use the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026).
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 samples or degraded RNA (see Appendix C, p.22) and inputs below 10 ng total RNA (see Appendix B, p.21).
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. **OPTIONAL**: For low input RNA this step can be extended to 1 hour incubation at 42 °C.
   ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

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

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. A reverse complement prevents the linker sequence from taking part in the hybridization.
NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, **EB**) for step 13 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. Thaw at 37 °C. If a precipitate is visible, incubate at 37 °C and mix until buffer components dissolve completely.

8	Add 15 µl Second Strand Synthesis Mix 1 (**SS1** ●) to the reaction. Mix well by pipetting, and seal the plate. **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). Incubate the reaction for 30 minutes at 25 °C. Quickly spin down the plate at room temperature before removing the sealing foil.
10	Prepare a mastermix containing 4 µl Second Strand Synthesis Mix 2 (**SS2** ●) and 1 µl Enzyme Mix 2 (**E2** ●). Mix well.
11	Add 5 µl of the **SS2** / **E2** mastermix per reaction. Mix well. **REMARK:** Use a pipette set to 40 µl for efficient mixing.
12	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 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.

13	Add 20 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature.
14	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).
15	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
16	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.
17	Add 72 µl 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 µl (**PS**) (see Appendix B, p.21 and Appendix C, p.22).
18	Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
19	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
20	Add 120 µl 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.
21	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.
22	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.
23	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.
24	Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
25	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 A, p.17 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!** 96-well PCR plate PCR sealing films	**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 } 11 -27 x see Appendix B, p.21 see Appendix C, p.22 72 °C, 1 min 10 °C, ∞	96-well magnetic plate 96-well PCR plate 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 may differ depending on the RNA used. A list of recommended cycle numbers for RNAs from a variety of organisms and tissues can be found at www.lexogen.com in the Frequently Asked Questions (FAQ) section of the QuantSeq webpage.
Lexogen offers a PCR Add-on Kit for Illumina (Cat. No. 020.96) which can be used for qPCR determination of the appropriate endpoint PCR cycle number on diluted cDNA samples. For details see Appendix A, p.17.
NOTE: At this point we recommend placing the Purification Beads (**PB**) for step 30 at room temperature to give them enough time to equilibrate.

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

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

28 Add 5 µl of the respective external Barcode Primer (**BC01-96**, in 96-well plate). **ATTENTION:** Spin down barcode plate before opening! Visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired barcodes. Avoid cross contamination! Reseal opened wells after usage to prevent cross contamination! Mix the PCR reaction well by pipetting. Seal the plate and quickly spin down to make sure all liquid is collected at the bottom of the well. **OPTIONAL:** If a qPCR is performed 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.17).

29 Conduct 11 -27 cycles of PCR (see Appendix B and C, p.21, p.22) with: Initial denaturation at 98 °C for 30 seconds, 11 - 27 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. ☞ 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**) must be properly resuspended before adding them to the reaction!

30 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, FFPE, or degraded RNA add only 27 µl Purification Beads (**PB**) (see Appendix B and C, p.21, 22).

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

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

33 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.

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

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

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

37 Add 120 µl 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.

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

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

40 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.

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

42 Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Do not transfer any beads. Libraries are now finished and ready for quality control (Appendix D, p.23), pooling (for multiplexing; see Appendix E, p.25), and cluster generation.

6. Short Procedure

ATTENTION: Spin down solutions before opening tubes or plates!

200 min Library Generation

☐	Mix 5 µl RNA and 5 µl **FS1** ●.	First Strand cDNA Synthesis
☐	Incubate at 85 °C for 3 min, then cool to 42 °C. Skip this step for low quality/low input/FFPE RNA.	
☐	Prepare a mastermix with 9.5 µl **FS2** ● and 0.5 µl **E1** ● per reaction.	
☐	Add 10 µl **FS2 / E1** mix per reaction.	
☐	Incubate for 15 min at 42 °C. **OPTIONAL:** May be increased to 1 h for low input RNA. ☞ Safe stopping point.	
☐	Add 5 µl **RS1** ○, mix well.	RNA Removal
☐	Incubate 10 min at 95 °C, then cool to 25 °C. Reduce to 5 min 95 °C for < 1 ng RNA input.	
☐	Add 5 µl **RS2** ○, mix well.	
☐	Add 15 µl **SS1** ●, mix well.	2nd Strand Synthesis
☐	Incubate 1 min at 98 °C, slowly ramp down to 25 °C (0.5 °C / sec).	
☐	Incubate 30 min at 25 °C.	
☐	Prepare a mastermix with 4 µl **SS2** ● and 1 µl **E2** ● per reaction.	
☐	Add 5 µl **SS2 / E2** mix per reaction, mix well.	
☐	Incubate 15 min at 25 °C. ☞ Safe stopping point.	
☐	Add 20 µl **PB** per reaction, mix well, incubate 5 min.	Purification
☐	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 72 µl **PS** (or 48 µl **PS** for low input, FFPE, or low quality 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**, 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.	

70 min Library Amplification

☐	Prepare a mastermix with 7 µl **PCR** ● and 1 µl **E3** ● per reaction, mix well.	PCR
☐	Add 8 µl **PCR** / **E3** premix to 17 µl of each purified library.	
☐	Add 5 µl **BC** (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 } 11 - 27 x (see p.21 and p.22) 72 °C, 1 min 10 °C, ∞. ☞ Safe stopping point. **ATTENTION:** Increase cycle number for low input RNA and samples with low mRNA content! (see p.21 and p.22)	
☐	Add 30 µl **PB** (or 27 µl **PB** for low quality/low input/FFPE RNA) per reaction, mix well, incubate 5 min.	Purification
☐	Place on magnet for 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**, 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.	

7. Appendix A: RNA Requirements

RNA Amount and Quality

In general, high quality mRNA-Seq data relies on high quality input RNA. However, as QuantSeq is a 3' mRNA-Seq protocol the quality of the RNA input is not as critical as for other RNA-Seq applications. With QuantSeq even lower quality RNA can yield good RNA-Seq results, therefore QuantSeq is highly suitable for FFPE samples. For further details on FFPE samples refer to Appendix C, p.22.

The amount of total RNA required for QuantSeq depends on the poly(A) RNA content of the sample in question. This protocol was tested extensively 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 libraries for single-end 50 nt (SR50) or paired-end 100 nt sequencing (PE100) 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) require protocol adjustments (see Appendix B, p.21).

With reduced total RNA input cycle numbers need to be adjusted accordingly (see Appendix B, p.21). For tissues with lower mRNA content (such as lung and heart) we recommend using 500 ng total RNA input. Low RNA input increases the likelihood of linker-linker artifacts and for samples using less than 5 ng input RNA an additional clean-up of the lane mix might be required. For QuantSeq REV (Cat. No. 016) we do not recommend RNA inputs below 10 ng.

The input requirements for your particular experiment may be different, as RNAs differ in their mRNA content. If RNA input is not sufficient, either due to naturally low poly(A) RNA content or degraded RNA, additional cycles of library amplification may be necessary. 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.

Lexogen also offers a PCR Add-on Kit for Illumina (Cat. No. 020.96), which can be used for qPCR assays, should you need to determine exact cycle numbers for your endpoint PCRs.

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 28 of the protocol. Dilute the double stranded library from step 25 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 (30 cycles or even more if little input material was used), 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 re-amplify 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. 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 more intensively 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. QuantSeq libraries generated from samples containing gDNA may have an increased number of intergenic reads or lower strandedness.
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 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.

8. Appendix B: PCR Cycles - Low RNA Input

Typically we recommend using 500 ng total RNA as starting material. If only limited total RNA is available, the input RNA amount can be reduced. Key parameters of libraries synthesized with Lexogen's QuantSeq kit using different input RNA amounts are shown in the table below. Total RNA inputs below 500 pg may already cause an increase in inserts with poly(T) sequences and/or Illumina linker sequences. Low RNA input (10 ng or less) requires protocol adjustments, such as reducing **PS** in step 17 to 48 µl. Even so, if the fraction of small fragments (library <150 bp, inserts <28 bp) becomes too prominent, an additional purification of the lane mix with 0.9 x **PB** may be necessary especially for less than 500 pg total RNA input (protocol in short: e.g., 50 µl lane mix plus 45 µl PB), mixing well, incubating 5 min at room temperature and following the protocol from step 31 on again). To avoid additional purification of the lane mix, we would recommend using at least 5 ng total RNA input. RNA inputs ≥ 200 ng are recommended to detect low abundant transcripts efficiently. For QuantSeq REV the lowest recommended input is 10 ng.

The table below depicts some key details when varying the RNA input amounts. Reference values were generated using Universal Human Reference RNA (UHRR). Other RNAs with lower mRNA content may require more PCR cycles. Please also refer to the QuantSeq Frequently Asked Questions (FAQs) page at www.lexogen.com for cycle number recommendations for other RNA sources (e.g., different mouse tissues, plants, or yeast). It is essential to avoid overcycling, indicated by a second high molecular weight peak between 1000 - 9000 bp in a Bioanalyzer trace, as this will bias your sequencing data. Take advantage of the PCR Add-On Kit (Cat. No. 020.96) and the qPCR assay as described on p.17, if you are uncertain about the cycle number for your endpoint PCR.

Input RNA (UHRR)	SS1 in step 8	PS used in step 17	Library*			Insert				Library yield		PCR cycles
			Start [bp]	End [bp]	Mean size*	Mean size	≥ 50 nt	≥ 100 nt	≥ 200 nt	ng/µl	nM	
2000 ng	std SS1	72 µl	122	1500	384	262	99 %	81 %	28 %	2.5	12.3	11
500 ng	std SS1	72 µl	122	1500	381	259	96 %	81 %	33 %	2.4	11.4	12
50 ng	std SS1	72 µl	122	1500	347	225	98 %	78 %	27 %	1.9	9.9	15
10 ng	std SS1	48 µl	122	1000	405	283	97 %	80 %	33 %	2.5	12.4	18
5 ng	std SS1	48 µl	122	1000	447	325	97 %	86 %	42 %	2.1	12.1	20
500 pg	std SS1	48 µl	122	1000	353	231	97 %	75 %	28 %	1.6	8.2	23

*All libraries are prepared with external Barcodes. Linker sequences are 122 bp including the 6 nt long external Barcodes.

ATTENTION: For input RNA amounts of 10 ng or less the amount of **PS** added in step 17 was reduced (48 µl instead of 72 µl) to prevent sequencing through linkers and poly(T) stretches.

Further adjustments for low input RNA (less than 10 ng) are skipping step 2 (recommended), extending step 4 to 1 hour (optional), reducing step 6 to 5 minutes 95 °C for less than 1 ng input RNA and reducing the amount of PB in step 30 to 27 µl (see FAQs at www.lexogen.com).

9. Appendix C: Low Quality RNA - FFPE

RNA isolated from Formalin-Fixed Paraffin Embedded (FFPE) samples is often heavily degraded. As QuantSeq is a 3' mRNA Seq protocol it is highly suitable for FFPE RNA.
For FFPE samples only minor protocol adjustments are required, such as skipping step 2 and reducing the addition of **PS** in step 17 to 48 µl as well as using only 27 µl PB in step 30.

As the RNA amount is often a limiting factor with FFPE samples, QuantSeq was tested with 500 pg - 50 ng FFPE or degraded RNA input. The table below comprises some results obtained from using different amounts of FFPE RNA input with a RIN of 2.8 (DV200 of 87 %). The DV200 value is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV200, the more degraded the RNA is. Other FFPE RNAs or RNAs with lower DV200 values may require more PCR cycles, hence we would strongly recommend using Lexogen's PCR Add-On Kit for Illumina (Cat. No. 020.96) and **taking advantage of the qPCR assay** as described on p.17.

Input RNA (UHRR)	PS used in step 17	Library*			Insert				Library yield		PCR cycles
		Start [bp]	End [bp]	Mean size*	Mean size	≥ 50 nt	≥ 100 nt	≥ 200 nt	ng/µl	nM	
50 ng	48 µl	122	500	220	98	56 %	12 %	1 %	1.5	12.3	18
10 ng	48 µl	122	500	233	111	84 %	36 %	3 %	1.2	8.7	24
500 pg	48 µl	122	500	240	118	79 %	37 %	5 %	0.4	3.1	27

*All libraries are prepared with external Barcodes. Linker sequences are 122 bp including the 6 nt long external Barcodes.

ATTENTION: FFPE RNA is degraded RNA, 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 31 on again may be necessary.

10. Appendix D: 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. Most UV-Vis spectrophotometers are not sensitive enough at these concentrations to accurately quantify NGS libraries and should be avoided.

Typical Results

QuantSeq libraries are intended for a high degree of multiplexing, and hence libraries do not need to be extensively amplified. 12 cycles of PCR amplification are usually enough when using 500 ng total RNA input. This will prevent overcycling and distorted expression values while at the same time providing enough material for quantification and subsequent cluster generation.

Typical concentrations are between 9.9 - 12.3 nM (1.9 - 2.5 ng/µl) for 50 ng (15 cycles) and 500 ng (12 cycles) input RNA, respectively, with most inserts being between 80 - 170 nts in length (see also Appendix B, p.21).

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.
Should your barcoded libraries be undercycled, you can reamplify them using Lexogen's PCR Add-on Kit for Illumina (Cat. No. 020.96). For more details please refer to Appendix A, p.17 as well as the PCR Add-on Kit Instruction Manual.

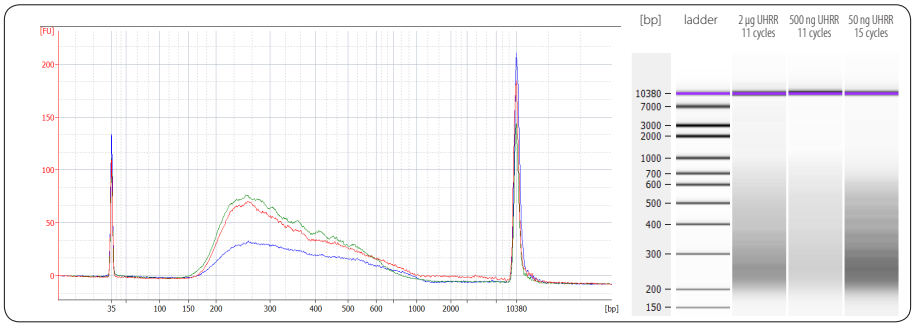

Figure 3. Bioanalyzer traces of QuantSeq libraries synthesized from 2 µg total RNA input (red trace) amplified with 11 cycles, 500 ng total RNA input (dark blue trace) amplified with 11 cycles (use 12 cycles for >10 nM yield), and 50 ng total RNA input (green trace) amplified with 15 cycles. Input RNA was Universal Human Reference RNA (UHRR).

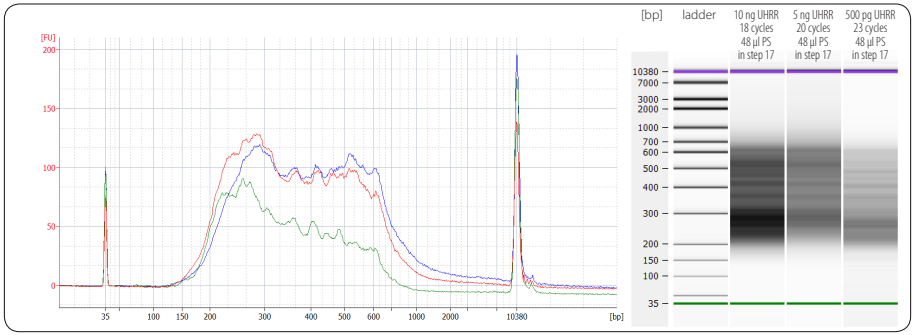

Figure 4. Bioanalyzer trace of QuantSeq libraries synthesized from 10 ng (red trace), 5 ng (blue trace), and 500 pg (green trace) total RNA input (Universal Human Reference RNA, UHRR) amplified with 18 cycles, 20 cycles, and 23 cycles, respectively. All three libraries were purified using 48 µl PS in step 17, which results in a better removal of sequences below 150 bp (inserts smaller than 28 bp).

11. Appendix E: 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 flowcell 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: AACAAG	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) include 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.

12. Appendix F: 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. See also our webpage for machine-specific loading instructions.
For paired-end sequencing we recommend using QuantSeq REV (Cat. No. 016.24, 016.96, 016.2x96).
A schematic representation of the two types of QuantSeq libraries (FWD Cat. No. 015 and REV Cat. No. 016) is shown below. The required sequencing primers are listed as well.

QuantSeq FWD Libraries with External Barcodes (Cat. No. 015.24, Cat. No. 015.96, Cat. No. 015.2x96)

External Barcodes (6 nt) are introduced during PCR (step 28).
For QuantSeq FWD libraries, Read 1directly corresponds to the mRNA sequence.

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

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

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

ATTENTION: We do not recommend paired-end sequencing for QuantSeq FWD (Cat. No. 015.24, Cat. No. 015.96, Cat. No. 015.2x96), as the quality of Read 2 would be very low due to the poly(T) stretch at the beginning of Read 2. For paired-end sequencing please use QuantSeq REV (Cat. No. 016.24, Cat. No. 016.96, Cat. No. 016.2x96).

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

QuantSeq REV Libraries with External Barcodes (Cat. No. 016.24, Cat. No. 016.96, Cat. No. 016.2x96)

External Barcodes (6 nt) are introduced during PCR (step 28).
For QuantSeq REV libraries, Read 1 corresponds to the cDNA sequence.

```
                                        5'-(Read 1 Custom Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-TTTTTTTTTTTTTTTTTT-Insert…
3'TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-AAAAAAAAAAAAAAAAAA-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: Custom Sequencing Primer (included):
5' CCCTACACGACGCTCTTCCGATCTTTTTTTTTTTTTTTTTTT 3'

ATTENTION: Do not use Multiplex Read 1 Sequencing Primer for QuantSeq REV (Cat. No. 016.24, Cat. No. 016.96, Cat. No. 016.2x96). Multiplex Read 1 Sequencing primer would result in a failed sequencing run as cluster calling would be impossible due to the poly(T) stretch.

ATTENTION: Do not mix **CSP** ● Version 2 and Read 1 Sequencing Primer! A primer mixture would result in low clusters calls and the resulting reads would be contaminated by poly(T) stretches.

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

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

Usage of the Custom Sequencing Primer CSP ●

For QuantSeq REV (Cat. No. 016.24, Cat. No. 016.96, Cat. No. 016.2x96) the Read 1 linker sequence is located at the 5' end of the oligodT primer. Here a Custom Sequencing Primer (**CSP** ● Version 2, included in the kit) is required for Read 1. The Custom Sequencing Primer covers the poly(T) stretch. Without the Custom Sequencing Primer cluster calling is not possible.
ATTENTION: Do not mix CSP ● and Read 1 Sequencing Primer! Do not mix **CSP** ● into HP10! A primer mixture would result in low clusters calls and the resulting reads would be contaminated by poly(T) stretches.

HiSeq 2000, HiSeq 2500 (CSP ● Version 2 added on cBot)

CSP Version 2 should be provided in a tube strip at 0.5 µM final concentration in a volume of 120 µl (final concentration 0.5 µM, to be diluted in HT1 = Hybridization buffer). Take 0.6 µl of 100 µM **CSP** Version 2 and add 119.4 µl of HT1 buffer per sequencing lane. Place the 8-tube strip into the cBot position labeled primers.

HiSeq 2500 (CSP ● Version 2 replaces HP10 in cBot Cluster Generation Reagent Plate)

Alternatively, **CSP** ● Version 2 can be placed directly into the cBot Cluster Generation Reagent Plate. **ATTENTION:** The standard Illumina Multiplex Read 1 Sequencing Primer solution HP10 (for V4 chemistry located in row 2) provided in the cBot Cluster Generation Reagent Plate has to be **REMOVED** first! The Illumina V4 chemistry cBot Cluster Generation Reagent Plate only has 8 rows filled. A simple trick is to have the empty rows facing towards you, this way if you want to use a **CSP** ● in lane 1, you have to remove the HP10 solution from well 1 (first one on the far left) of the 2nd row, rinse the well a couple of times with HT1 and then add the diluted **CSP** ● Version 2. For this take 1.25 µl of 100 µM **CSP** ● Version 2 and add 248.75 µl of HT1 buffer per sequencing lane. The **CSP** ● should be at 0.5 µM final concentration in a volume of 250 µl (final concentration 0.5 µM, to be diluted in HT1 = Hybridization buffer). **ATTENTION:** Do not add the CSP to the Standard Illumina Multiplex Read 1 Sequencing Primer = HP10 solution! Always use fresh HT1 and add the **CSP** ● / HT1 dilution to the empty and rinsed well.

HiSeq 2500 - Rapid Run

Add 12.5 µl of 100 µM **CSP** ● Version 2 to 2487.5 µl HT1 = Hybridization buffer, resulting in a total volume of 2.5 ml and a final **CSP** ● concentration of 0.5 µM. In a rapid run, both lanes will use the same sequencing primer. It is not possible to run the two lanes with different sequencing primers.

MiSeq

Clustering is performed on the machine, not on the c-Bot. The MiSeq uses a reservoir of 600 µl with 0.5 µM sequencing primer final concentration, i.e., 3 µl of 100 µM **CSP** ● Version 2 in 597 µl HT1.

HiSeq 3000, HiSeq 4000 (CSP ● Version 2 replaces HP10 in cBot Cluster Generation Reagent Plate)

Usage of a custom sequencing primer is currently not supported on HiSeq 3000 and 4000 machines. A work around as described for the HiSeq2500 (**CSP** ● Version 2 **REPLACES** HP10 in the cBot Cluster Generation Reagent Plate) is possible though. **ATTENTION:** Do not add the **CSP** ● Version 2 to the HP10 solution! A primer mixture would result in low clusters calls and the resulting reads would be contaminated by poly(T) stretches. Always use fresh HT1 and add the **CSP** ● Version 2 / HT1 dilution to the empty and rinsed well.

13. Appendix G: 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 is available in two read orientations: QuantSeq FWD (Cat. No. 015) 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. To pinpoint the exact 3′ end, longer read lengths may be required. Read 1 directly reflects the mRNA sequence.
In QuantSeq REV (Cat. No. 016), the Read 1 linker sequence is located at the 5′ end of the oligodT primer. For Read 1, a Custom Sequencing Primer (included in the kit) has to be used. With QuantSeq REV it is possible to exactly pinpoint the 3′ end during Read 1. The reads generated here during Read 1 reflect the cDNA sequence, so they are in a strand orientation opposite to the genomic reference. For paired-end sequencing we strongly recommend using QuantSeq REV (Cat. No. 016).

For more detailed information please refer to https://www.lexogen.com/quantseq-data-analysis.

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.

Trimming

As second strand synthesis is based on random priming, there may be a higher proportion of er-

rors at the first nucleotides of the insert due to non-specific hybridization of the random primer to the cDNA template. These mismatches can lead to a lower percentage of mappable reads when using a stringent aligner such as TopHat2, in which case it may be beneficial to trim these nucleotides. For QuantSeq FWD (Cat. No. 015) the first 12 nucleotides of Read 1 need to be removed. Alternatively, a less stringent aligner (e.g., STAR Aligner) could be used with the number of allowed mismatches being set to 14. While trimming the first nucleotides can decrease the number of reads of suitable length, the absolute number of mapping reads may increase due to the improved read quality. Reads, which are too short or have generally low quality scores should be removed from the set.
While single-read sequencing does not require any trimming using QuantSeq REV (Cat. No. 016), paired-end sequencing may require the first 12 nucleotides of Read 2 to be trimmed. Alternatively, also here the STAR Aligner could be used with the number of allowed mismatches being set to 16 for paired-end reads.

Alignment

At this point the filtered and trimmed reads can be aligned with a short read aligner to the reference genome.
STAR aligner or TopHat2 can be used for mapping **QuantSeq FWD** (Cat. No. 015) data. The reads may not land in the last exon and span a junction hence splice-aware aligners should be used.
For **QuantSeq REV** (Cat. No. 016) we do not recommend using TopHat2, since there is hardly a need to search for junctions. Nearly all sequences will originate from the last exon and the 3' untranslated region (UTR). In case of no detected junction, TopHat2 may run into difficulties. Hence, Bowtie2 or BWA can be used for mapping in this case.

Annotation

Mapping only the 3' end of transcripts requires an annotation which covers the 3' untranslated region (UTR) accurately. Conservative annotations might decrease the power of correct gene quantification after mapping, especially in case of QuantSeq REV (Cat. No. 016). For some gene annotations it might be an advantage to extend the 3' UTR annotation further downstream in order to assign the mapped read correctly.

Please visit our website (www.lexogen.com) for an up-to-date table of suggested species-specific annotations and comments. More information about the principal data analysis can be found under www.lexogen.com.

Integrated Data Analysis Pipeline on Bluebee Platform

Each QuantSeq kit comes with a code for free data analysis on Bluebee platform. Please visit www.bluebee.com/quantseq for more information and to access the data analysis pipeline. To login enter the code received with the kit.

For further inquiries please contact info@lexogen.com.

14. Appendix H: Revision History

Revision date/ Publication No.	Change	Page
015UG009V0215	In Short procedure: remove from magnet after EB addition.	16
015UG009V0214	Rinse with RNAse-free water after RNAZap usage!	8
	QuantSeq-Flex First Strand Synthesis Module for longer insert size. Removed SS1 dilution.	11, 12
	Thaw SS1 at 37 °C. Check for precipitates. Mix until buffer components dissolve completely.	12
	Visually inspect fill levels of BC Plate.	14
	Lowest recommended input RNA amount set to 10 ng for QuantSeq REV.	17, 21
	Changes to Input RNA amount Table and Figure 3 and 4. SS1 dilution removed.	21, 24
015UG009V0213	Indication of safe stopping points.	11, 12, 13, 15, 16
015UG009V0212	Optional: Step 4 (RT) may be increased to 1 h @ 42 °C for low input RNA.	11,16, 21
	Reduce step 6 to 5 minutes at 95 °C for RNA inputs below 1 ng total RNA.	12,16, 21
	Pierce or cut open new barcode sealing.	14
	2000 ng input RNA example and SS1 dilution added to input RNA table and Figure 3.	21, 24
	Link to https://www.lexogen.com/quantseq-data-analysis.	29
015UG009V0211	CSP concentration changed to 100 µM.	6, 28
	Increased RT temperature from 37 °C to 42 °C to prevent internal oligodT priming.	11, 16
	Changes to RS1/RS2 for increased stability.	12
	Option to generate longer insert sizes through SS1 dilution.	12, 16
	Endpoint PCR set at 33 % of the maximum qPCR fluorescence.	18
	Re-Amplification primer in PCR Add-on Kit.	18, 24
	Usage of the Custom Sequencing Primer CSP Version 2 (100 µM) for QuantSeq REV.	28
015UG009V0210	Changes to Logo and listing of kits. Consistency changes (Fig.1, PS in preparation table post PCR).	1, 5, 14
	Changes to Fig.2; Listing of Purification Module as separate Item in Kit Contents Table.	6
	Recommendation on SYBR Green I, and more details on dilutions for qPCR.	7, 18
	Removal of CSP recommendations for NextSeq500 and NextSeq550.	27
015UG009V0200	Renaming of QuantSeq T-fill to QuantSeq REV, and QuantSeq 015 to QuantSeq FWD.	1, 4
	Figure 1: Including Sequencing orientation for QuantSeq FWD and QuantSeq REV.	5
	Explicit instructions to centrifuge at room temperature.	11, 12
	Recommendations for Low Quality RNA - FFPE.	11, 13, 15
	Appendix C: Low Quality RNA - FFPE, Table with cycle number recommendations.	21
	Usage of the Custom Sequencing Primer for QuantSeq REV.	27
	Alignment with Bowtie 2 or BWA for faster mapping.	29
015UG009V0112	PCR Add-on Kit and Instructions for PCR endpoint determination.	17
015UG009V0111	Minor User Guide changes (mainly semicolons). Capital letters on Reference Card.	
June 11th 2014	Adjusted Figure 1 to match PCR and cDNA synthesis color coding; included barcode in Figure 1-	5
	Feasibility of QuantSeq for low quality RNA input.	17
	Updated Table and Figures (libraries synthesized from 500 ng- 500 pg input RNA (UHRR)).	20, 22
	Revision History Table.	27
March 24th 2014	Initial Release 015UG009V0100.	

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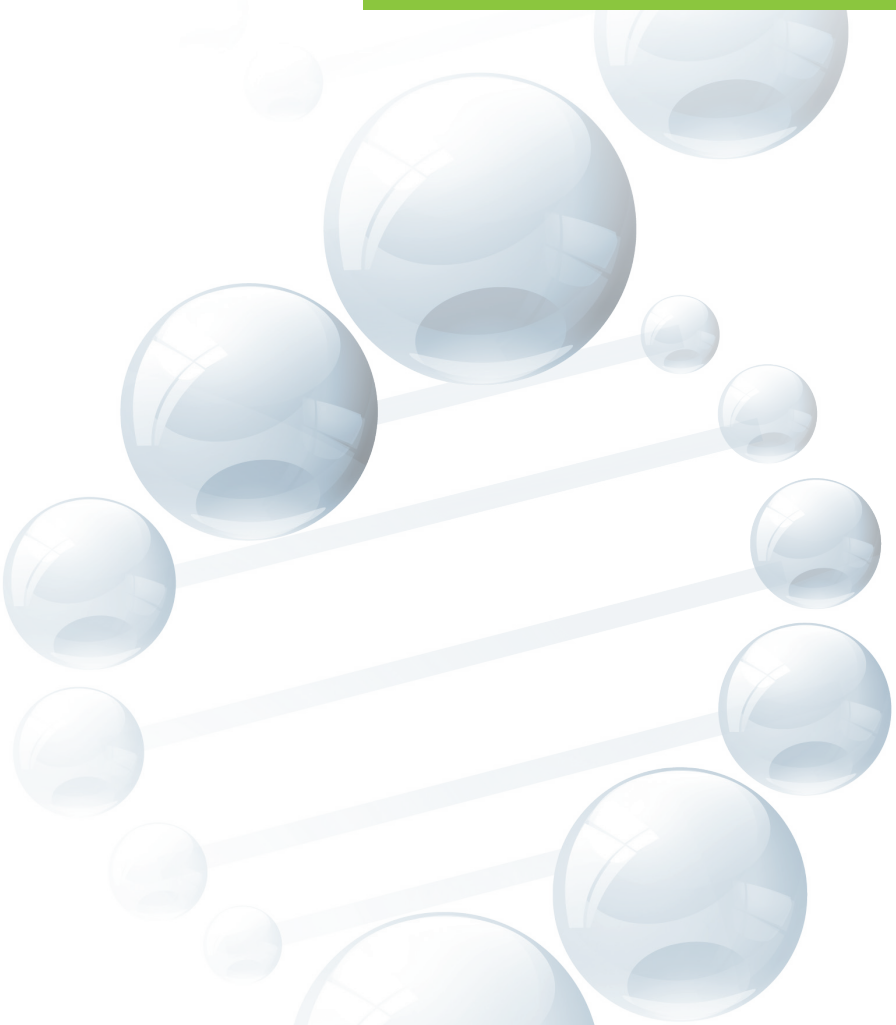

QuantSeq 3′ mRNA-Seq Library Prep Kit · User Guide

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