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QUANT™ SEQ

Sequencing that counts

3' mRNA-Seq Library Prep Kit FWD HT

User Guide

Catalog Numbers:
015 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (FWD))
015 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (FWD) HT including i5 Dual Indexing Add-on Kit (5001-5004))
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, 050, 051 (Spike-In RNA Variant Controls: SIRV-Set 1, SIRV-Set 2, SIRV-Set 3)
026 (QuantSeq-Flex First Strand Synthesis Module for Illumina)
028 (QuantSeq-Flex Second Strand Synthesis Module V2 for Illumina)
047 (i5 Dual Indexing Add-on Kit for QuantSeq/SENSE for Illumina (5001-5004))

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ATTENTION: Barcodes rearranged!

015UI125V0101

Initial release QuantSeq 3′ mRNA-Seq FWD HT Kit

Please note that the kit is based on the upgraded QuantSeq 3′ mRNA-Seq version (kits after February 17th, 2017) including the following changes:

- **First Strand cDNA Synthesis (p.11): Do not cool the FS2/E1 mastermix (step 3) and have the RNA/FS1 samples at 42 °C when adding the FS2/E1 mastermix (step 4) to avoid mishybridization. Mix properly by pipetting. Do not forget to shortly spin down the samples at room temperature before and after adding the FS2/E1 mastermix.**
- New arrangement and renaming of the barcode plate to improve the nucleotide balance → i7 Index Plate (7001-7096), unique set of barcodes – no overlap with Illumina-specific indices (BC05 removed). An evaluation tool to check the color balance of index subsets is available on the Lexogen website.
- Barcode 00 (BC00) in PCR Add-on Kit renamed to P7 Primer 7000.
- RNA Removal (p.12): No more addition of RS2 solution (previously step 7) → one step less in the protocol; RS1 renamed to RS.
- Second Strand Synthesis (p.12): Addition of SS1 in step 7 (previously step 8) is reduced to 10 µl (instead of 15 µl).
- Purification (p.13): Adjusted volumes for the purification steps (16 µl PB in step 12 instead of 20 µl in previously step 13 and 56 µl PS in step 16 instead of 72 µl in previously step 17).
- Dual indexing for up to 384 unique barcode combinations introduced
- qPCR endpoint determination using only 1.7 µl template and set to 50 % of the maximum fluorescence. Subtract 3 cycles from determined cycle number for the endpoint PCR when using 10x as much template.

For more details please have a look at the Revision History at the end of this User Guide or contact us under support@lexogen.com or +43 1 345212 41.

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LITERATURE CITATION
For any publication using this product, please refer to it as Lexogen's QuantSeq™ 3′ mRNA-Seq Kit FWD HT.

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1. Overview

Lexogen's QuantSeq kit FWD HT provides a high-throughput 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. The sequences obtained are close to the 3' end of the transcripts.
QuantSeq FWD HT (Cat. No. 015.384) is a high-throughput version (384 preps) with optional dual indexing to prevent sample mix-ups and to increase the number of available indices. The Read 1 linker sequence is located at 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 QuantSeq FWD HT (Cat. No. 015.384). 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.

QuantSeq FWD HT 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 is compatible with automation. Multiplexing of libraries can be carried out using up to 96 i7 indices (provided in a 96-well plate) and four perfectly balanced i5 indices (provided in microtubes), resulting in 384 unique index combinations.

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.21).
Library generation is initiated by oligo(dT) 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, SR75, SR100).
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 I, p.34 for a schematic representation of the finished library). i5 and i7 multiplexing indices are included in the QuantSeq FWD HT kit (Cat. No. 015.384) and are introduced during this step. For index sequences refer to Appendix H, p.32. More details on index read-out on various Illumina platforms can be found in Appendix I, p.34.

Library quantification can be performed with standard protocols (see Appendix F, p.28).
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 J, p.36. A QuantSeq bioinformatics pipeline has been integrated on the Bluebee genomics analysis platform and each purchased QuantSeq kit includes a code for free data analysis on the Bluebee platform. For more details visit our webpage at www.lexogen.com.

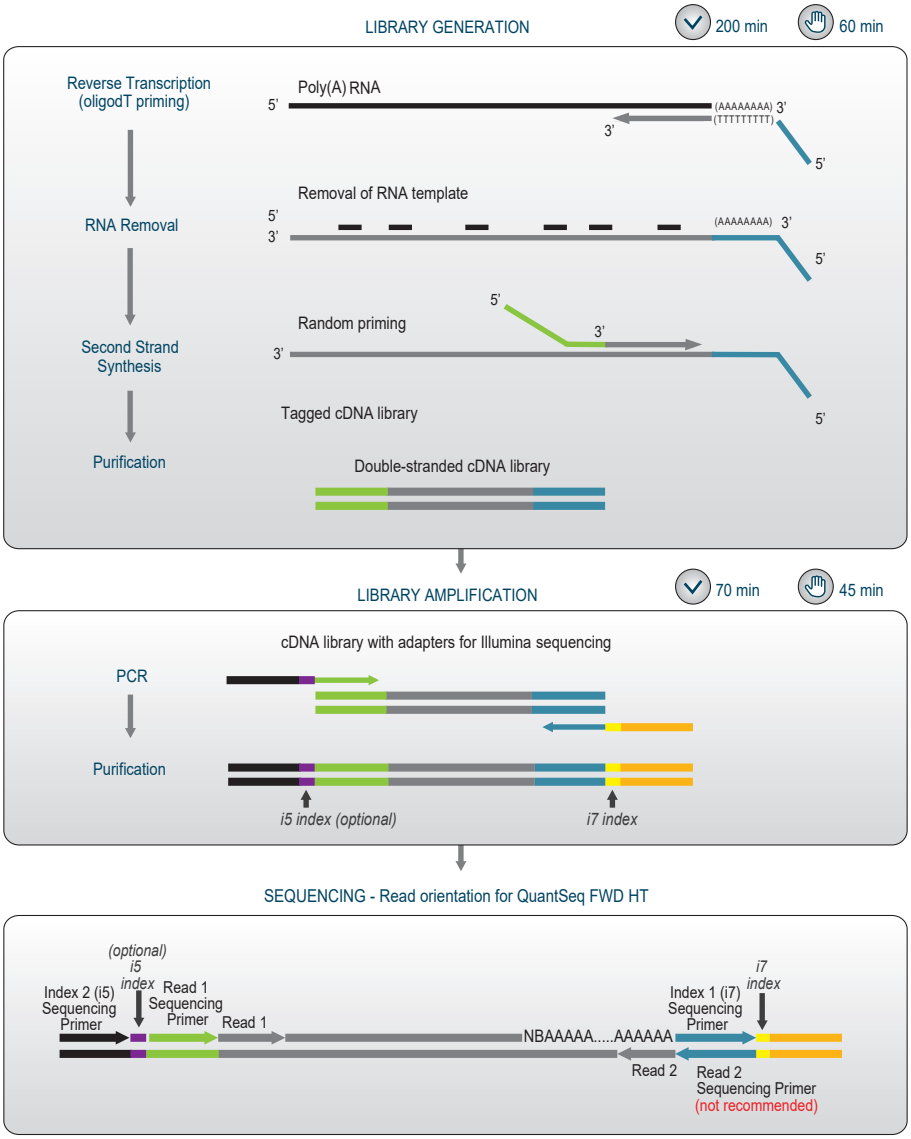


Figure 1. Schematic overview of the QuantSeq FWD HT library preparation workflow (Cat. No. 015.384). Illumina P5 adapters (Read 1) are shown in green and Illumina P7 adapters (Read 2) are shown in blue. Read 1 reflects the mRNA sequence. Paired-end sequencing is not recommended for QuantSeq FWD HT (Cat. No. 015.384). i7 and optional i5 indices are introduced during the PCR amplification step and allow multiplexing of up to 384 samples in one NGS lane.

2. Kit Components and Storage Conditions

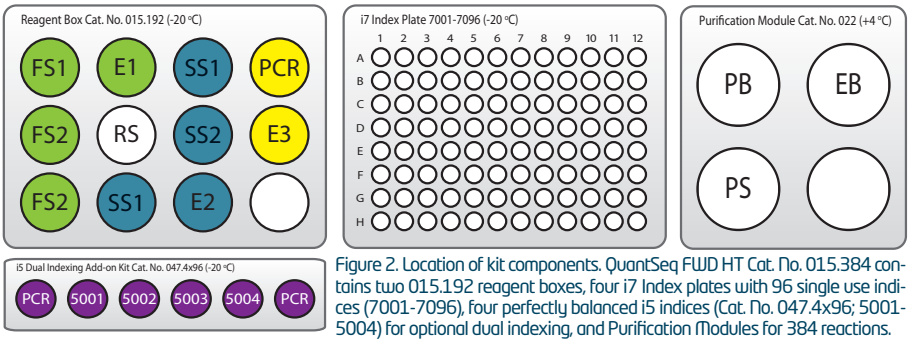


Figure 2. Location of kit components. QuantSeq FWD HT Cat. No. 015.384 contains two 015.192 reagent boxes, four i7 Index plates with 96 single use indices (7001-7096), four perfectly balanced i5 indices (Cat. No. 047.4x96; 5001-5004) for optional dual indexing, and Purification Modules for 384 reactions.

Kit Component	Tube Label	Volume* 384 preps	Storage
First Strand cDNA Synthesis Mix 1	FS1	2,112 µl	-20 °C
First Strand cDNA Synthesis Mix 2	FS2	4,013 µl	-20 °C
Enzyme Mix 1	E1	212 µl	-20 °C
RNA Removal Solution	RS	2,112 µl	-20 °C
Second Strand Synthesis Mix 1	SS1	4,224 µl	-20 °C
Second Strand Synthesis Mix 2	SS2	1,690 µl	-20 °C
Enzyme Mix 2	E2	423 µl	-20 °C
PCR Mix	PCR	2,957 µl	-20 °C
Enzyme Mix 3	E3	423 µl	-20 °C
i7 Index Plate (96-well plate)		5 µl / reaction	-20 °C
i5 Dual Indexing Add-on Kit (Cat. No 047.4x96) included in the kit			
PCR Mix	PCR	2,957 µl	-20 °C
i5 Index 01	5001	528 µl	-20 °C
i5 Index 02	5002	528 µl	-20 °C
i5 Index 03	5003	528 µl	-20 °C
i5 Index 04	5004	528 µl	-20 °C
Purification Modules (4x Cat. No. 022.96) included in the kit			
Purification Beads	PB	21,542 µl	+4 °C
Purification Solution	PS	36,326 µl	+4 °C
Elution Buffer	EB	53,645 µl	+4 °C / -20 °C

*including 10 % surplus

Upon receiving the QuantSeq kit, store the Purification Module (Cat. No. 022.96), containing **PB**, **PS**, and **EB** at +4 °C and the rest of the kit in a -20 °C freezer. **REMARK: EB** can be stored either at +4 °C or -20 °C. Before use, check the contents of **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 (for washing of Purification Beads, **PB**).
- Optional: SYBR Green I (Sigma-Aldrich, Cat. No. S9430), 10,000x 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 1,000 µl volumes.
- Calibrated multi-channel pipettes for handling 1 µl to 200 µl volumes.
- Thermocycler.
- UV-spectrophotometer to quantify RNA.
- Ice bath or ice box, ice pellets, benchtop cooler (-20 °C for enzymes).

Labware

- Suitable certified ribonuclease-free 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.

Optional Equipment

- Automated microfluidic electrophoresis station (e.g., 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).

The complete set of material, reagents, and labware necessary for RNA extraction and quality control is not listed. Consult Appendix A and B, p.21 and p.23 for more information on RNA quality.

Consult Appendix F, p.28 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 RNaseZap residue with RNase-free water after usage! Residues of RNaseZap may damage the RNA.
- Protect all reagents and your RNA samples from RNases on your skin by wearing a clean lab coat and fresh gloves. Change gloves after making contact with equipment or surfaces outside of the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

Bead Handling

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

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

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

General

- Unless explicitly mentioned, all steps including centrifugations, should be carried out at room temperature (RT) between 20 °C and 25 °C. Results may be negatively impacted if the protocol is performed at temperatures outside of this range. While reaction set-up is often performed at RT, incubation temperatures are explicitly defined, and must be strictly adhered to.
- To further increase reproducibility and to avoid cross contamination a centrifugation step should be performed after incubations at elevated temperatures and before removing the sealing foil from PCR plates (e.g., step 2).
- Ensure that adequate volumes of all reagents and the necessary equipment are available before beginning the protocol.
- Perform all pipetting steps with calibrated pipettes, and always use fresh tips. Pipette carefully to avoid foaming as some solutions contain detergents.
- Thaw all necessary buffers at room temperature or as indicated in the preparation tables at the beginning of each step of the detailed protocol. Mix reagents well by vortexing or pipetting repeatedly and centrifuge briefly with a benchtop centrifuge to collect contents before use.
- Keep Enzyme Mixes at -20 °C until 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

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. 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 10 use a pipette set to 15 µl or 30 µl, respectively, to ensure proper mixing.

Pipetting and Handling of (Viscous) Solutions

- Enzyme Mixes, **SS1** ●, **PB**, and **PS** are viscous solutions which require care to pipette accurately. 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, 9, and 25 of the QuantSeq HT 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 HT kit include a 10 % surplus.

Automation

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

5. Detailed Protocol

5.1 Library Generation

Preparation

First Strand cDNA Synthesis	RNA Removal	Second Strand Synthesis	Purification
FS1 ● – thawed at RT **FS2** ● – thawed at RT **E1** ● – keep on ice or at -20 °C	**RS** ○ – 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 PCR sealing films Plate centrifuge 85 °C, 3 min 42 °C, 15 min	Thermocycler PCR sealing films Plate centrifuge 95 °C, 10 min cool down to 25 °C	Thermocycler PCR sealing films Plate centrifuge 98 °C, 1 min, then cool to 25 °C (0.5 °C/sec) 25 °C, 30 min 25 °C, 15 min	96-well magnetic plate 96-well PCR plate PCR sealing films

First Strand cDNA Synthesis - Reverse Transcription

An oligo(dT) primer containing an Illumina-compatible sequence at its 5' end is hybridized to the RNA and reverse transcription is performed. QuantSeq FWD HT libraries are intended for a high degree of multiplexing.

1. Mix up to 5 µl of your RNA (typically 500 ng, see also Appendix A to D, p.21-26) with 5 µl First Strand cDNA Synthesis Mix 1 (**FS1** ●) in a PCR plate. 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. 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, see Appendix H, p.31).
2. Denature the RNA / **FS1** mix for 3 minutes at 85 °C in a thermocycler and then cool down to 42 °C. **REMARK:** Leave the reactions on the thermocycler at 42 °C until step 4. **ATTENTION:** Skip this step for FFPE samples or degraded RNA (see Appendix D, p.26) and inputs below 10 ng total RNA (see Appendix C, p.24).
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. **ATTENTION:** Do not cool the mastermix!
4. Quickly spin down the denatured RNA / **FS1** mix, carefully remove the sealing foil, and place onto the thermocycler again. 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 (≤10 ng) 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 (**RS** ○) directly to the first strand cDNA synthesis reaction. Mix well and reseal the plate using a fresh foil. **REMARK:** Use a pipette set to 15 µl for efficient mixing.
6	Incubate 10 minutes at 95 °C, then cool down to 25 °C. Spin down the plate at room temperature and carefully remove the sealing foil. **ATTENTION:** Reduce this step for RNA inputs below 1 ng total RNA to 5 minutes at 95 °C (see Appendix C, p.24).

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 12 at room temperature to give it 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.

7	Add 10 µl Second Strand Synthesis Mix 1 (**SS1** ●) to the reaction. Mix well by pipetting, and seal the plate. **REMARK:** Use a pipette set to 30 µl for efficient mixing.
8	Incubate the plate for 1 minute at 98 °C in a thermocycler, and slowly cool down to 25 °C by setting the ramp speed to 10 % (0.5 °C/second). Incubate the reaction for 30 minutes at 25 °C. Quickly spin down the plate at room temperature before removing the sealing foil.
9	Prepare a mastermix containing 4 µl Second Strand Synthesis Mix 2 (**SS2** ●) and 1 µl Enzyme Mix 2 (**E2** ●). Mix well.
10	Add 5 µl of the **SS2** / **E2** mastermix per reaction. Mix well. **REMARK:** Use a pipette set to 30 µl for efficient mixing.
11	Incubate the reaction at 25 °C for 15 minutes. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

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

12	Add 16 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature.
13	Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear (depends on the strength of your magnet).
14	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
15	Add 40 µl of Elution Buffer (**EB**), remove the plate from the magnet, and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
16	Add 56 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly, and incubate for 5 minutes at room temperature. **ATTENTION:** For low input RNA (≤10 ng), FFPE, or degraded RNA add only 48 µl **PS** (see Appendix C, p.24 and Appendix D, p.26).
17	Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
18	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
19	Add 120 µl of 80 % EtOH, and incubate the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.
20	Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely as traces of ethanol can inhibit subsequent PCR reactions.
21	Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.
22	Add 20 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
23	Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
24	Transfer 17 µl of the clear supernatant into a fresh PCR plate. Make sure not to transfer any beads. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point. See Appendix E, p.27 for qPCR options.

5.2. Library Amplification

The library is amplified to add the complete adapter sequences required for cluster generation and to generate sufficient material for quality control and sequencing. Here either single indexing (i7 Index Primer, follow protocol 5.2.1 Single Indexing, p.14-16) or dual indexing PCR (i7 and i5 Index Primer, follow protocol 5.2.2 Dual Indexing, p.16-18) can be used. PCR volumes and post PCR purification differ between those two indexing options and are discussed in detail in the respective subsections.

5.2.1 Single Indexing (i7 only)

Single indexing PCR (i7 indices only) enables multiplexing and unique indexing of 96 libraries. i7 indices are provided in a 96-well plate (**7001-7096**).

Preparation

PCR	Purification
PCR ● – thawed at RT **E3** ● – keep on ice or at -20 °C **7001-7096 (i7 Indices)** – thawed at RT; in 96-well plate; **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
96-well PCR plate PCR sealing films Plate centrifuge Thermocycler 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec } 11 -22x 72 °C, 30 sec see Appendix C, p.24 see Appendix D, p.26 72 °C, 1 min 10 °C, ∞	96-well magnetic plate 96-well PCR plate Plate centrifuge PCR sealing films

PCR

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

ATTENTION: Cycle numbers may differ depending on the RNA used. 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 E, p.27.

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

25	Prepare a mastermix containing 7 µl of PCR Mix (**PCR** ●) and 1 µl Enzyme Mix 3 (**E3** ●) per reaction.
26	Add 8 µl of this **PCR / E3** mastermix to 17 µl of the eluted library.
27	Add 5 µl of the respective i7 index (**7001-7096**, in 96-well plate). Mix well by pipetting. Seal the PCR plate and quickly spin down to make sure all liquid is collected at the bottom of the well. **ATTENTION:** Spin down i7 Index Plate before opening! Visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired indices. Avoid cross contamination! Reseal opened wells of the i7 primer plate after usage to prevent cross contamination! **OPTIONAL:** If a qPCR is performed, use 5 µl of the P7 Primer (**7000** ●) included in the PCR Add-on Kit for Illumina (Cat. No. 020.96) at this step for the qPCR and the respective i7 primer (**7001-7096**, in 96-well plate) for the subsequent endpoint PCR (see Appendix E, p.27).
28	Conduct 11 - 22 cycles of PCR (see Appendix C and D, p.24-26, or see Appendix E, p.27 for qPCR) with: Initial denaturation at 98 °C for 30 seconds, 11 - 22 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!

29	Add 30 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature. **ATTENTION:** For low input (≤10 ng), FFPE, or degraded RNA add only 27 µl Purification Beads (**PB**) (see Appendix C and D, p.24-26).
30	Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
31	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
32	Add 30 µl of Elution Buffer (**EB**), remove the plate from the magnet, and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
33	Add 30 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly, and incubate for 5 minutes at room temperature.
34	Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
35	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

36	Add 120 µl of 80 % EtOH, and incubate the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.
37	Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.
38	Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.
39	Add 20 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet, and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
40	Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
41	Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Do not transfer any beads. Libraries are now finished and ready for quality control (Appendix F, p.28), pooling (for multiplexing; see Appendix H, p.32), and cluster generation. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

5.2.2 Dual Indexing (i7 and i5)

The following protocol replaces the single indexing PCR and post PCR purification described on p.14-15. Dual indices (i5 and i7) are introduced and the final PCR volume for dual indexing is 35 µl. If a qPCR is intended to determine the exact cycle number for endpoint PCR, use the PCR Add-on Kit (Cat. No. 020.96) and follow the instructions of the Instruction Manual (020IM064) or see Appendix E, p.27. The same qPCR approach can be used no matter if single or dual indexing is selected for endpoint PCR.

Preparation

PCR		Purification	
PCR ● (from **i5 Dual Indexing Add-on Kit**)	– thawed at RT	**PB**	– stored at +4°C
E3 ●	– keep on ice or at -20 °C	**PS**	– stored at +4°C
5001-5004 ● (from **i5 Dual Indexing Add-on Kit**)	– thawed at RT; **spin down!**	**80 % EtOH**	– provided by user **prepare fresh!**
7001-7096 (i7 Index Plate)	– thawed at RT; 96-well plate; **spin down before opening!**	**EB**	– stored at +4°C
Thermocycler 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec } 11- 22x see Appendix C, p.24 see Appendix D, p.26 72 °C, 1 min 10 °C, ∞ 96-well PCR plate PCR sealing films Plate centrifuge		96-well magnetic plate 96-well PCR plate Plate centrifuge PCR sealing films	

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.

For qPCR determination of the appropriate endpoint PCR cycle number, use the PCR Add-on Kit (Cat. No. 020.96) and follow the instructions of 020IM064 or see Appendix E, p.27. The single indexing PCR (i7 only) of the PCR Add-on Kit and the dual indexing PCR (i5 and i7) run with the same efficiency, hence there is no need to exchange any solutions.
NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, **EB**) for step 30 at room temperature to give them enough time to equilibrate.

25 Prepare a mastermix containing 7 µl PCR Mix from the i5Dual Indexing Add-on Kit (**PCR** ●) and 1 µl Enzyme Mix 3 (**E3** ●) from the QuantSeq basic kit per reaction. **ATTENTION:** Do not use the PCR Mix (**PCR** ●) from the basic kits if dual indexing is intended!

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

27 Add 5 µl of the respective i5 Index Primer (**5001-5004** ●, provided in microtubes with the i5 Dual Indexing Add-on Kit). Mix well by pipetting.

28 Add 5 µl of the respective i7 Index Primer (**7001-7096,** provided in 96-well plate, supplied with the basic kits). Mix well by pipetting. Seal the PCR plate and quickly spin down to make sure all liquid is collected at the bottom of the well. **ATTENTION:** Spin down the i7 index plate before opening! Pierce or cut open the sealing foil of the wells containing the desired indices. Avoid cross contamination! Reseal opened wells of the i7 primer plate after usage to prevent cross contamination!

29 Conduct 11 - 22 cycles of PCR (see recommendations in Appendices C and D, p.24-26 or determine by qPCR, see Appendix E, p.27) with: Initial denaturation at 98 °C for 30 seconds, 11 - 22 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**) may have settled and must be properly resuspended before adding them to the reaction. The following purification replaces the post PCR purification for single indexing described on p.15-16.

30 Add 35 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature. **ATTENTION:** For QuantSeq libraries generated from low RNA input (≤10 ng), from FFPE, or degraded RNA, add only 31.5 µl **PB** (see Appendix C and D, p.24-26).

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 reprecipitate 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 incubate the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.
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:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.
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. Make sure not to transfer any beads.
43	At this point, the libraries are finished and ready for for quality control (Appendix F, p.28), pooling (for multiplexing see Appendix H, p.32), and cluster generation. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

6. Short Procedure

6.1 Single Indexing (i7 only)

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 input (≤10 ng)/low quality/FFPE RNA. **ATTENTION:** Keep samples on thermocycler at 42 °C.	
☐	Prepare a mastermix with 9.5 µl **FS2** ● and 0.5 µl **E1** ● per reaction, mix well. Do not cool!	
☐	Add 10 µl **FS2 / E1** mix per reaction, mix well.	
☐	Incubate for 15 min at 42 °C. **OPTIONAL:** May be increased to 1 h for low input RNA (≤10 ng). ☞ Safe stopping point.	
☐	Add 5 µl **RS** ○, mix well.	RNA Removal
☐	Incubate 10 min at 95 °C, then cool to 25 °C. Reduce to 5 min at 95 °C for ≤1 ng RNA input.	
☐	Add 10 µl **SS1** ●, mix well.	Second 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, mix well.	
☐	Add 5 µl **SS2 / E2** mix per reaction, mix well.	
☐	Incubate 15 min at 25 °C. ☞ Safe stopping point.	
☐	Add 16 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 56 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.	
☐	Air dry beads for 5 - 10 min. **ATTENTION:** Do not let the beads dry too long!	
☐	Add 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.	
☐	Place on magnet for 2 - 5 min, transfer 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.	

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 i7 primer (**7001-7096**, from the 96-well plate), mix well. **ATTENTION:** Reseal opened index wells after usage!	
☐	PCR: 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec } 11 - 22x (see p.24-26) 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.24-26)	
☐	Add 30 µl **PB** (or 27 µl **PB** for low input (≤10 ng)/low quality/FFPE RNA) per reaction, mix well, incubate 5 min.	Purification
☐	Place on magnet for 2 - 5 min, remove, and discard supernatant.	
☐	Add 30 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.	
☐	Add 30 µl **PS**, mix well, incubate 5 min at RT.	
☐	Place on magnet for 2 - 5 min, remove and discard supernatant.	
☐☐	Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.	
☐	Air dry beads for 5 - 10 minutes. **ATTENTION:** Do not let the beads dry too long!	
☐	Add 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.	
☐	Place on magnet for 2 - 5 min, transfer 15 - 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.	

6.2 Dual Indexing (i7 and i5)

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 input (≤10 ng)/low quality/FFPE RNA. **ATTENTION:** Keep samples on thermocycler at 42 °C.	
☐	Prepare a mastermix with 9.5 µl **FS2** ● and 0.5 µl **E1** ● per reaction, mix well. Do not cool!	
☐	Add 10 µl **FS2 / E1** mix per reaction, mix well.	
☐	Incubate for 15 min at 42 °C. **OPTIONAL:** May be increased to 1 h for low input RNA (≤10 ng). ☞ Safe stopping point.	
☐	Add 5 µl **RS** ○, mix well.	RNA Removal
☐	Incubate 10 min at 95 °C, then cool to 25 °C. Reduce to 5 min at 95 °C for ≤1 ng RNA input.	
☐	Add 10 µl **SS1** ●, mix well.	Second 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, mix well.	
☐	Add 5 µl **SS2 / E2** mix per reaction, mix well.	
☐	Incubate 15 min at 25 °C. ☞ Safe stopping point.	
☐	Add 16 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 56 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.	
☐	Air dry beads for 5 - 10 min. **ATTENTION:** Do not let the beads dry too long!	
☐	Add 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.	
☐	Place on magnet for 2 - 5 min, transfer 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.	

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 **i5** (in microtubes, from the i5 Dual Indexing Add-on Kit) for each reaction, mix well.	
☐	Add 5 µl **i7** (from the 96-well plate) for each reaction, mix well. **ATTENTION:** Reseal opened index wells after usage!	
☐	PCR: 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec } 11 - 22x (see p.24-26) 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.24-26)	
☐	Add 35 µl **PB** (or 31.5 µl **PB**) for low input (≤10 ng)/low quality/FFPE RNA) per reaction, mix well, incubate 5 min.	Purification
☐	Place on magnet for 2 - 5 min, remove, and discard supernatant.	
☐	Add 30 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.	
☐	Add 30 µl **PS**, mix well, incubate 5 min at RT.	
☐	Place on magnet for 2 - 5 min, remove and discard supernatant.	
☐☐	Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.	
☐	Air dry beads for 5 - 10 minutes. **ATTENTION:** Do not let the beads dry too long!	
☐	Add 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.	
☐	Place on magnet for 2 - 5 min, transfer 15 - 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.	

7. Appendix A: General RNA Requirements

Potential Contaminants

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

Genomic DNA Contamination

Depending on the RNA extraction protocol used, samples may also contain significant amounts of genomic DNA (gDNA), which is indistinguishable from RNA on a spectrophotometer. Furthermore, as many of the dyes used in RNA microfluidics assays stain single-stranded nucleic acids 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. While DNAse I treatment my be advisable for FFPE RNA, in general we do not recommend DNase treatment, as the extended incubation with divalent cations can lead to RNA hydrolysis and decrease RNA integrity. If samples must be DNase treated, heat inactivation should be avoided, and the enzyme should be deactivated by other means such as phenol / chloroform extraction or silica column purification.

RNA Integrity

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 removes mt-rRNAs, such as Lexogen's RiboCop rRNA Depletion Kit (Cat. No. 037), can be used before starting the QuantSeq library preparation if it is essential to remove mt-rRNA transcripts.

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 Variants), 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. SIRVs are available in three sets, SIRV-Set 1 (Cat. No 025.03) contains the mixes E0, E1, and E2 of the isoform module, SIRV-Set 2 (Cat. No 050.01 and 050.03) provides the Isoform Mix E0 only, whereas SIRV-Set 3 (Cat. No 051.01 and 051.03) has the SIRV Isoform Mix E0 in a mixture with the ERCC RNA Spike-in controls (Ambion Inc., see below).

ERCC RNA Spike-in Controls

To enable the hypothesis-neutral calculation of strandedness, to assess internal oligo(dT) 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 oligo(dT) 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: RNA Input Quality and Amount

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 D, p.26.

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 Human Brain Reference RNA (HBRR)). Typical inputs of 500 ng total RNA generate high quality libraries for single-read 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 (≤10 ng) require protocol adjustments (see Appendix C, p.24).

With reduced total RNA input, cycle numbers need to be adjusted accordingly (see Appendix C, p.24). 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 a clean-up of the lane mix might be required.

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 (see also Appendix C, p.24). Overcycling of libraries - indicated by a second high molecular weight peak between 1,000 - 9,000 bp in a Bioanalyzer trace - should be prevented as this may lead to distortions in transcript abundance estimation 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 the exact cycle numbers for your endpoint PCRs. For more details please refer to Appendix E, p.27.

9. Appendix C: PCR Cycles (incl. Low RNA Input)

Key parameters of libraries synthesized with Lexogen's QuantSeq kit using different input RNA amounts of Universal Human Reference RNA (UHRR) are shown in the table below. 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. Low RNA input (10 ng or less) requires protocol adjustments (see bottom of the page). Total RNA inputs below 500 pg may already cause an increase in inserts with poly(T) sequences and/or Illumina linker sequences. If the fraction of small fragments (library ≤150 bp, inserts ≤28 bp) becomes too prominent, an additional purification of the lane mix with 0.9x **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 30 (single PCR, p.15) 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.

The table below depicts some key details when varying the RNA input amounts. Reference values were generated using Universal Human Reference RNA (UHRR).

Input RNA (UHRR)	Step 6: RNA Removal 95°C	Step 16: PS Addition	Library*			Insert				Library Yield		PCR Cycles
			Start [bp]	End [bp]	Mean Size*	Mean Size	≥ 50 nt	≥ 100 nt	≥ 200 nt	ng/µl	nM	
2,000 ng	10 min	56 µl	132	2,000	456	324	97 %	80 %	31 %	2.0	10.2	11
500 ng	10 min	56 µl	132	2,000	364	232	98 %	78 %	27 %	1.8	9.8	12
100 ng	10 min	56 µl	132	2,000	350	218	97 %	74 %	21 %	2.1	11.3	14
50 ng	10 min	56 µl	132	2,000	389	257	96 %	70 %	20 %	2.4	12.7	15
10 ng	10 min	48 µl	132	2,000	350	218	96 %	70 %	24 %	2.6	14.1	18
5 ng	10 min	48 µl	132	2,000	365	233	97 %	75 %	28 %	3.2	15.7	19
500 pg	5 min	48 µl	132	2,000	335	203	89 %	67 %	21 %	1.4	8.0	22

*All libraries were prepared with dual indexing. Linker sequences are 132 bp including 6 nt long i5 and i7 indices, respectively. For single Indexing (i7 only) adapter sequences are 122 bp.

ATTENTION: For input RNA amounts of 10 ng or less, we recommend reducing the amount of **PS** added in step 16 (48 µl instead of 56 µl) to prevent sequencing through linkers and poly(T) stretches.

Further adjustments for low input RNA (≤10 ng) are skipping step 2 (recommended), extending step 4 to 1 hour (optional), reducing step 6 to 5 minutes at 95 °C for less than 1 ng input RNA, and reducing the amount of **PB** in step 29 to 27 µl for single indexing PCR and in step 30 to 31.5 µl for dual indexing PCR, respectively (see also FAQs at www.lexogen.com).

Other RNAs with lower mRNA content may require more PCR cycles. It is essential to avoid overcycling, indicated by a second high molecular weight peak between 1,000 - 9,000 bp in a Bio-

analyzer 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 in Appendix E, p.27 to determine the exact cycle number for your endpoint PCR.

In the following table some reference values (cycles numbers and approximate yields) for 500 ng input RNA from other RNA sources are depticted:

500 ng Input RNA from	Library Yield		PCR Cycles
	ng/µl	nM	
Universal Human Reference RNA (UHRR)	1.8	9.8	12
Human Brain Reference RNA (HBRR)	2.0	13.8	13
Mm heart	1.9	10.5	13
Mm brain	2.9	15.6	13
Mm liver	1.3	6.7	12
Mm kidney	2.3	12.2	12
Mm spleen	1.4	8.0	13
Mm lung	2.6	15.5	14
Mm embryonic stem cells	1.3	7.5	11
Mm myoblast	0.9	5.2	12
Mm fibroblast	1.0	5.6	14
Mm myoblast progenitors	2.1	11.5	11
Mm neural progenitors	1.2	7.0	12
Arabidopsis thaliana	1.7	9.4	13
Tomato seeds	1.7	9.4	16
Fungi RNA	1.24	7.1	13
Yeast RNA (Sc)	1.2	7.7	12
Drosophila melanogaster	1.6	7.9	13

Mm: Mus musculus; Sc: Saccharomyces cerevisiae

Please also refer to the QuantSeq Frequently Asked Questions (FAQs) page at www.lexogen.com for continuous updates on other RNA sources.

10. Appendix D: 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 16 to 48 µl as well as
- using only 27 µl **PB** in step 29 for single indexing PCR and 31.5 µl in step 30 for dual indexing PCR, respectively (see FAQs at www.lexogen.com).

Further optional adjustments for low FFPE RNA inputs may be included such as:

- Extend the reverse transcription time in step 4 to 1 h (≤10 ng).
- Reduce the RNA removal time in step 6 to 5 min at 95 °C (≤1 ng).

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 mouse (Mm) brain FFPE RNA input with a RIN of 1.8 (DV_{200} of 51 %). The DV_{200} is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV_{200}, the more degraded the RNA is. Other FFPE RNAs or RNAs with lower DV_{200} values may require more PCR cycles, hence we strongly recommend using Lexogen's PCR Add-on Kit for Illumina (Cat. No. 020.96) and **taking advantage of the qPCR assay** as described in Appendix E, p.27.

Input RNA (Mm brain FFPE RNA)	Step 6: RNA Removal 95°C	PS used in step 16	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	10 min	48 µl	132	600	264	132	96 %	52 %	5 %	2.3	14.2	15
10 ng	10 min	48 µl	132	600	265	133	95 %	56 %	5 %	2.1	13.1	18
500 pg	5 min	48 µl	132	600	266	134	85 %	53 %	4 %	1.2	7.5	22

*All libraries were prepared with dual indexing. Linker sequences are 132 bp including 6 nt long i5 and i7 indices, respectively.

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 generates a lot of linker-linker products despite the above-mentioned protocol changes, an additional purification of the lane mix with 0.9 x **PB** (e.g., 50 µl lane mix plus 45 µl **PB**, incubating 5 minutes at room temperature, and following the protocol from step 30 (single PCR, p.15) on again) may be necessary.

NOTE: FFPE RNA may also be contaminated with fragmented DNA, which may result in an overestimation of inserted RNA and/or in a high number intergenic reads in NGS samples. For FFPE RNA it may therefore be advisable to perform a DNase I treatment or at least distinguish between RNA and DNA when quantifying your input material.

NOTE: Up-to-date best-practice recommendations on FFPE / low quality and low RNA input samples can be found at www.lexogen.com under QuantSeq Frequently Asked Questions.

11. Appendix E: qPCR and Reamplification

The mRNA content determines the PCR cycles needed during the final PCR amplification step. Hence, we strongly recommend taking advantage of the qPCR assay. Over- or undercycling may bias your sequencing results (transcript abundance estimation and library quantification).

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 the P7 Primer (**7000** ●) included in the PCR Add-on Kit for Illumina (Cat. No. 020.96) instead of an i7 index in step 27 of the single indexing PCR protocol (see p.15). Dilute the double-stranded library from step 24 to 19 µl by adding 2 µl Elution Buffer (**EB**) in order to have enough template for qPCR and endpoint PCR. Add 1.7 µl of the cDNA into a PCR reaction containing 7 µl **PCR** ●, 5 µl **7000** ●, and 1 µl **E** ● from the PCR Add-on Kit. 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). Fill up the total PCR reaction volume to 30 µl with **EB**. 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 (1:100 SYBR Green I dilution, diluted in DMSO) 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 or even 50 cycles if little input material was used; include a no template control!). Determine the fluorescence value at which the fluorescence reaches the plateau. Calculate where the fluorescence is at 50 % of the maximum and determine at which cycle these 50 % of fluorescence are reached. As in the endpoint PCR 10x more cDNA will be used compared to the qPCR, three cycles can be subtracted from the determined cycle number. This is the cycle number you should use for the endpoint PCR using the remaining 17 µl of the template. There is no need to purify or analyze the overcycled PCR reaction on a Bioanalyzer. **EXAMPLE:** 500 ng total RNA input was used for generating libraries. When inserting 1.7 µl of the cDNA into a qPCR, the cycle number determined in the overcycled qPCR (50 % of the maximum fluorescence) was 15 cycles. The remaining 17 µl of the template (i.e.,10 times more cDNA than in the qPCR, hence -3 cycles) should be amplified with 12 cycles. **ATTENTION:** The qPCR approach described here is valid regardless if dual or single indexed endpoint PCRs are intended.

Reamplification of i7 Indexed Libraries (i7 only)

Lexogen's PCR Add-on Kit also contains a Reamplification Primer (**RE** ○) that can be used to reamplify single indexed (i7) libraries to get enough material for sequencing if they were undercycled. For details please refer to the PCR Add-on Kit (Cat. No. 020.96) Instruction Manual.

ATTENTION: Do not use **7000** ● for the reamplification of i7 indexed libraries! This will lead to a loss of indices and to a mixed and not assignable sequence pool in the 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 i7 indexed, amplified PCR libraries. For reamplification of dual indexed libraries, contact Lexogen at info@lexogen.com.

12. Appendix F: 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, shape, and the size distribution of libraries.

Quality Control Methods

The analysis of a small volume of the amplified library with microcapillary electrophoresis has become the *de facto* standard for many NGS laboratories and generates information regarding library concentration and size distribution. Several electrophoresis platforms are available from various manufacturers. For low- to medium-throughput applications, we recommend the Agilent Bioanalyzer 2100 and High Sensitivity DNA chips (Agilent Technologies, Inc.). For high throughput applications instruments such as the Fragment Analyzer (Advanced Analytical Technologies, Inc.), LabChip GX II (Perkin Elmer), or 2200 TapeStation (Agilent Technologies, Inc.) are recommended. Typically, 1 µl of a QuantSeq library produced according to the directions in this manual is sufficient for analysis. Depending on the minimum sample loading requirements for each instrument, 1 µl of the finished library may be diluted to the required volume (e.g., 2 µl sample for TapeStation and 10 µl for LabChip GX II).

More accurate library quantification can be achieved with custom or commercially available qPCR assays. With these assays, the relative or absolute abundance of amplifiable fragments contained in a finished QuantSeq library is calculated by comparing Cq values to a set of known standards. While delivering a more accurate quantification, these assays do not supply the user with information regarding library size distribution. Unwanted side-products such as linker-linker artifacts are not discernible from the actual library in the qPCR assay as both will be amplified. Hence it is highly recommended to combine such an assay for quantification with microcapillary electrophoresis analysis for library size distribution.

If microcapillary electrophoresis platforms and qPCR machines are not available, very basic quality control can also be performed by separating a small aliquot of the library on a polyacrylamide or agarose gel. Library quantification can also be performed with an inexpensive benchtop fluorometer using one of several commercially available assays, e.g., Qubit dsDNA HS assay. 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 12.7 - 9.8 nM (2.4 - 1.8 ng/µl) for 50 ng (15 cycles) and 500 ng (12 cycles) input RNA, respectively, with most inserts being between 110 - 200 nt in length (see also Appendix C, p.24).

A shorter side-product caused by priming of the second strand synthesis oligo on the oligo(dT) 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 1,000 - 9,000 bp) is an indication of overcycling. This could occur if cycle numbers are increased too much to compensate for lower input material. Prevent overcycling by using the qPCR assay as described in Appendix E, p.27.
Lexogen's PCR Add-on Kit for Illumina (Cat. No. 020.96) offers the option to reamplify undercycled single indexed libraries. For more details please refer to Appendix E, p.27 as well as the PCR Add-on Kit Instruction Manual (020IM064). For reamplification of dual indexed libraries contact Lexogen at info@lexogen.com.

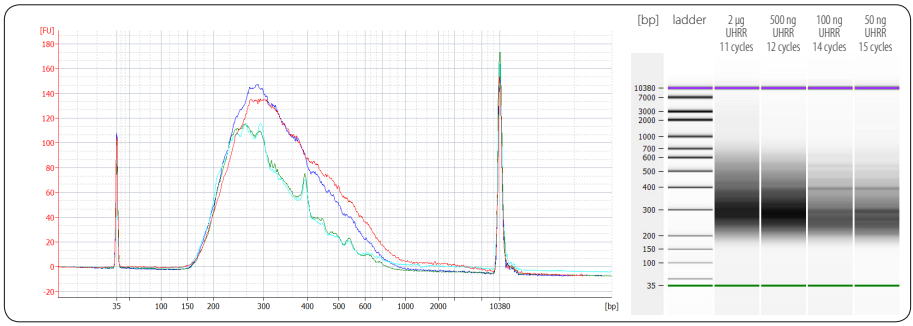


Figure 3. Bioanalyzer trace of QuantSeq FWD HT libraries synthesized from 2 µg (red trace), 500 ng (dark blue trace), 100 ng (green trace) and 50 ng (light blue trace) total RNA input (Universal Human Reference RNA, UHRR) amplified with 11 cycles, 12 cycles, 14 cycles, and 15 cycles, respectively. Dual indexing was used for all libraries.

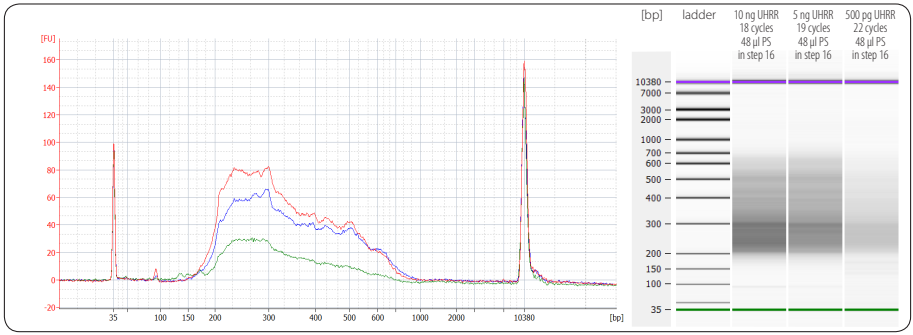


Figure 4. Bioanalyzer trace of QuantSeq FWD HT libraries synthesized from 10 ng (red trace), 5 ng (blue trace), and 500 pg (green trace) total RNA input (Universal Human Reference RNA, UHRR) amplified with 17 cycles, 18 cycles, and 22 cycles, respectively. Step 2 was skipped for all samples and for 500 pg input RNA step 6 was reduced to 5 min at 95 °C. For all three libraries only 48 µl PS was added in step 16, which results in a better removal of sequences below 150 bp (inserts smaller than 28 bp). Dual indexing PCR was used for all libraries and 31.5 µl PB were used in step 30.

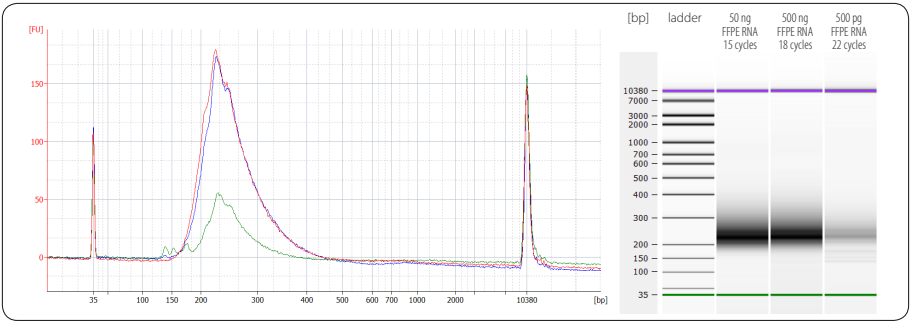


Figure 5. Bioanalyzer traces of QuantSeq FWD HT libraries synthesized from 50 ng (red trace), 10 ng (dark blue trace), and 500 pg (green trace) mouse (Mm) brain FFPE RNA (RIN 1.8, DV_{200} 51 %). All libraries were prepared with the recommendationds for FFPE RNA input. 500 pg FFPE RNA libraries already contain some artifacts below 150 bp which should be removed before sequencing, e.g., by repurifying the lane mix (see Appendix D, p.26).

13. Appendix G: Modulating Insert Sizes

The QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026.96) can be used to increase insert sizes. For details regarding oligo(dT) primed first strand synthesis using the QuantSeq-Flex First Strand Synthesis Module, please refer to the corresponding QuantSeq-Flex User Guide (015UG058).

In short: **FS1** ● and **FS2** ● from the basic QuantSeq FWD kit are exchanged with **FS1x** ●, **FS2x** ●, and **dT** ● from the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026.96). Due to buffer differences and protocol changes, longer inserts can be generated.

Examples of such library length increases are depicted in Figure 6.

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

*All libraries were prepared from 500 ng Universal Human Reference RNA (UHRR) with dual indexing. Linker sequences are 132 bp including 6 nt long i5 and i7 indices, respectively. FS1x, FS2x, and oligo dT (dT) are components from the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026.96). FS1 and FS2 are from the QuantSeq FWD HT basic kit.

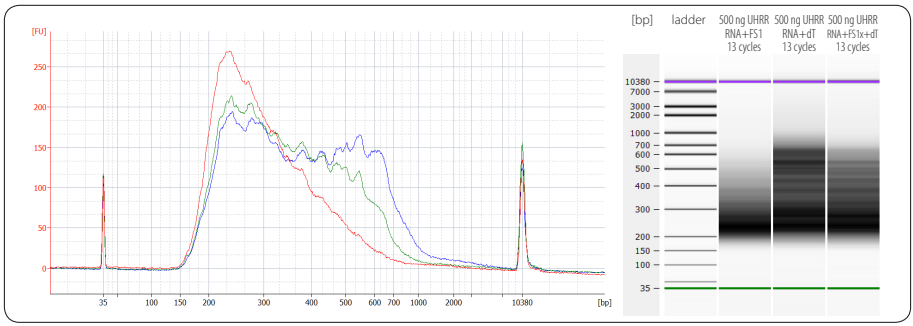


Figure 6. Bioanalyzer traces of QuantSeq FWD HT libraries with library size modulation. The shortest insert sizes are generated with the basic QuantSeq kit components, i.e., denaturing of RNA in FS1 (red trace, standard protocol). With the QuantSeq-Flex First Strand Synthesis Module and denaturing RNA and oligo(dT) (dT) only, before adding FS1x, FS2x, and E1 the longest average insert sizes are generated (dark blue trace). With the QuantSeq-Flex First Strand Synthesis Module and denaturing RNA, FS1x, and dT before adding FS2x and E1 the average insert size can be increased (green trace). Input RNA was 500 ng Universal Human Reference RNA (UHRR) for all libraries. Dual indexing with 13 cycles of final library amplification was used for all libraries.

14. Appendix H: Multiplexing

QuantSeq libraries are designed for a high degree of multiplexing. i5 and i7 indices are introduced during the PCR amplification step.

i7 Indices

i7 indices allowing up to 96 samples to be sequenced per lane on an Illumina flow cell are included in the kit (i7 Index Plate, 96-well plate). i7 Indices are 6 nt long and require an additional index-specific sequencing reaction (Index 1 Read).

	1	2	3	4	5	6	7	8	9	10	11	12
A	7001: CAGCGT	7009: TCAGGA	7017: TCTTAA	7025: TTTATG	7033: AGATAG	7041: CTCTCG	7049: GTGCCA	7057: AGTACT	7065: AAGCTC	7073: GACATC	7081: GCAGCC	7089: CGCGGA
B	7002: GATCAC	7010: CGGTTA	7018: GTCAGG	7026: AACGCC	7034: TTGGTA	7042: TGACAC	7050: TCGAGG	7058: ATAAGA	7066: GACGAT	7074: CGATCT	7082: ACTCTT	7090: CCTGCT
C	7003: ACCAGT	7011: TTAACT	7019: ATACTG	7027: CAAGCA	7035: GTTACC	7043: AAGACA	7051: CACTAA	7059: GGTGAG	7067: TCGTTC	7075: CGTCGC	7083: TGCTAT	7091: GCGCTG
D	7004: TGCACG	7012: ATGAAC	7020: TATGTC	7028: GCTCGA	7036: CGCAAC	7044: ACAGAT	7052: GGTATA	7060: TTCCGC	7068: CCAATT	7076: ATGGCG	7084: AAGTGG	7092: GAACCT
E	7005: ACATTA	7013: CCTAAG	7021: GAGTCC	7029: GCGAAT	7037: TGGCGA	7045: TAGGCT	7053: CGCCTG	7061: GAAGTG	7069: AGTTGA	7077: ATTGGT	7085: CTCATA	7093: TTCGAG
F	7006: GTGTAG	7014: AATCCG	7022: GGAGGT	7030: TGGATT	7038: ACCGTG	7046: CTCCAT	7054: AATGAA	7062: CAATGC	7070: AACCGA	7078: GCCACA	7086: CCGACC	7094: AGAATC
G	7007: CTAGTC	7015: GGCTGC	7023: CACACT	7031: ACCTAC	7039: CAACAG	7047: GCATGG	7055: ACAACG	7063: ACGTCT	7071: CAGATG	7079: CATCTA	7087: GGCCAA	7095: AGGCAT
H	7008: TGTGCA	7016: TACCTT	7024: CCGCAA	7032: CGAAGG	7040: GATTGT	7048: AATAGC	7056: ATATCC	7064: CAGGAC	7072: GTAGAA	7080: AACAAG	7088: AGACCA	7096: ACACGC

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

In general, we recommend processing a minimum of 8 samples, using a complete set of eight i7 indices for multiplexing (e.g., **7001-7008**). However, if fewer indices are required care should be taken to always use indices which give a well-balanced signal in both lasers (red and green channels) for each nucleotide position. All columns (1 - 12) and rows (A - H) fulfill these criteria. An evaluation tool to check the color balance of index subsets is available at www.lexogen.com. The individual libraries within a lane should be mixed at an equimolar ratio to ensure this balance.

REMARK: If an 8 nt i7 index (Index 1) needs to be entered into an Illumina sample sheet, e.g., if QuantSeq libraries are multiplexed with 8 nt indexed libraries from other NGS-kit suppliers, add two nucleotides from the Illumina adapter sequence to the 3' end of the index. **EXAMPLE: 7001** would become CAGCGT**AT**, **7002** would become GATCAC**AT** and so on. These additional nucleotides are identical for all indices as they are derived from the Illumina adapter.

i5 Indices

The i5 Dual Indexing Add-on Kit (Cat. No. 047) contains 4 perfectly balanced i5 Indices (**5001-5004** ●) suitable for every Illumina sequencing machine. Each nucleotide (A, C, G, and T) is present at each position of the 6 nt long index. This is of particular importance for NextSeq and MiniSeq sequencing platforms, where only a two-channel read-out is used: T is labeled with green, C is labeled with red, A is a mixture of a different red and green, and G remains unlabeled.

i5 read-out is performed during the index 2-specific sequencing reaction. Depending on the Illumina sequencing machine, there are differences when and how Index 2 is read out (for details see Appendix I, p.34). **ATTENTION:** This affects the way the i5 indices have to be entered into the corresponding sample sheets. A dual indexed sequencing run on the MiniSeq, NextSeq, HiSeq 3000, or HiSeq 4000 performs the Index 2 Read after the Read 2 resynthesis step, hence here the reverse complement of the index is read. Details on how to enter the i5 indices on the respective sequencing machines are shown in the table below (see also Appendix I, p.34).
EXAMPLE: **5001** is read as CGCCAT on a MiSeq but as ATGGCG on a NextSeq.
REMARK: Some Illumina sample sheets may require 8 nt to be entered for Index 2. In this case, add two nucleotides from the Illumina adapter sequence to the 3' end of the index e.g., **5001** becomes CGCCAT**AC** on a MiSeq and ATGGCG**GT** on a NextSeq, respectively.

Index 2 (i5) Oligo	i5 Bases for Sample Sheet	
	MiSeq, HiSeq 2000/2500	MiniSeq, NextSeq, HiSeq 3000/4000
5001 ●	CGCCAT (AC)	ATGGCG (GT)
5002 ●	ATTTTA (AC)	TAAAAT (GT)
5003 ●	GCAACG (AC)	CGTTGC (GT)
5004 ●	TAGGGC (AC)	GCCCTA (GT)

Nucleotides in brackets (AC) or (GT) are derived from the Illumina adapters and are not actually part of the index. They are listed only for completeness if the sample sheet requires 8 nt to be entered.

The individual libraries within a lane should be mixed at an equimolar ratio to ensure the perfect nucleotide balance. **EXAMPLE:** For 4 libraries use all four i5 indices (**5001-5004** ●). For 8 libraries use **5001** ● for 2 libraries in combination with two different i7 indices (from the i7 Index Plate, **7001-7096**), **5002** ● for 2 libraries in combination with two different i7 Indices (from the i7 Index Plate, **7001-7096**), **5003** ● for 2 libraries in combination with two different i7 Indices (from the i7 Index Plate, **7001-7096**), and **5004** ● for 2 libraries in combination with two different i7 Indices (from the i7 Index Plate, **7001-7096**).

Each i5 index can be combined with any of the i7 indices provided within the i7 Index Plate (**7001-7096**) of the QuantSeq 3' mRNA-Seq Kit FWD HT for Illumina (Cat. No. 015.384). By combining the four different i5 indices with the 96 different i7 indices, 384 different index combinations can be achieved.

15. Appendix I: Sequencing*

General

The amount of library loaded onto the flow cell 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 flow cell. Machine-specific loading instructions can be found at www.lexogen.com under Frequently Asked Questions. Platform-specific index read-outs are described in more detail in the following section.

ATTENTION: We do not recommend paired-end sequencing for QuantSeq FWD HT (Cat. No. 015.384), as the quality of Read 2 would be very low due to the poly(T) stretch at the beginning of Read 2.

Single Indexing (i7 only) - All Illumina Platforms

Index 1 (i7) is read out directly after Read 1. i7 Indices (6 nt) are provided in the i7 Index Plate, **7001-7096**.

After Read 1, Index 1 (i7) is sequenced.

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

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

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

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

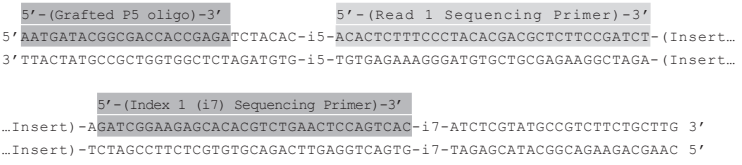
Dual Indexing (i7 and i5)

Dual Index read-out differs depending on the Illumina platform and flow cell (single-read or paired-end flow cell) used. Single-read flow cells and paired-end flow cells are available for HiSeq systems. MiniSeq, NextSeq, and MiSeq systems only include paired-end flow cells. The following sections describe the dual indexing read-out in more detail.

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

Dual Indexing on Single-Read Flow Cell - HiSeq 3000 and HiSeq 4000

Index 1 (i7) is read out directly after Read 1. i7 Indices (6 nt) are provided in the i7 Index Plate, **7001-7096**. The i5 indices are part of the i5 Dual Indexing Add-on Kit (Cat. No. 047). Seven additional chemistry-only cycles are required to read the i5 index, as here the grafted P5 oligo is used to initiate Index 2 (i5) read-out. This step uses the resynthesis mix during the Index 2 Read process.



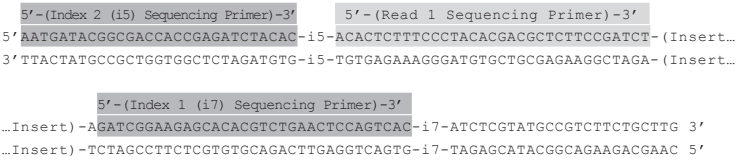
Read 1: Multiplexing Read 1 Sequencing Primer (not supplied):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

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

Index 2 Read (i5): Grafted P5 oligo on Flow Cell (not supplied):
5' AATGATACGGCGACCACCGAGA 3'

Dual Indexing on Single-Read Flow Cell - HiSeq 2000 and HiSeq 2500

Index 1 (i7) is read out directly after Read 1. i7 Indices (6 nt) are provided in the i7 Index Plate, **7001-7096**. The i5 indices are part of the i5 Dual Indexing Add-on Kit (Cat. No. 047). Here, Index 2 sequencing primer (included in HP9) is required for Index 2.
After Read 1, Index 1 (i7) and Index 2 (i5) are sequenced.



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

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

Index 2 Read (i5): Multiplexing Index 2 (i5) Sequencing Primer (not supplied):
5' AATGATACGGCGACCACCGAGATCTACAC 3'

16. Appendix J: 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 HT (Cat. No. 015.384) 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. **ATTENTION:** Paired-end sequencing is not recommended for QuantSeq FWD HT (Cat. No. 015.384).

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.

Demultiplexing

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

Trimming

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

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.

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. 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.

More information about the principal data analysis can be found under https://www.lexogen.com/quantseq-data-analysis.

Integrated Data Analysis Pipeline at Bluebee

Each purchased QuantSeq kit includes a code for free data analysis including differential expression (DE) analysis using the Bluebee platform. 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.

17. Appendix K: Revision History

Revision date/ Publication No.	Change	Page
015UG110V0102 Jul. 31, 2017	Front page and SIRVs text updated.	0, 22
	After denaturing (step 2), leave the reactions on the thermocycler at 42 °C until step 4.	11, 19-20
	Dry beads only at room temperature after ethanol washes.	13, 16, 18-20
	Added note that up-to-date recommendations can be found on the QuantSeq FAQ webpage.	26
	Consistency changes.	
015UG110V0101 Mar. 8, 2017	Do not cool the FS2/E1 mastermix.	11
	Safe stopping points. Fixed typo.	16, 18 - 20, 26
015UG110V0100 Feb. 7, 2017	Release of QuantSeq FWD HT Cat. No. 015.384 with included (optional) i5 Dual Indexing Add-on Kit.	
	Barcode Plate (BC) was rearranged for improved balance and renamed to i7 Index Plate (7001-7096). Previous BC05: TAATCG replaced by 7025: TTTATG to avoid overlap with Illumina-specific indices.	4-6, 14-17, 19-20, 31-35
	RS1, renamed to RS. No more RS2 solution, hence one step less in the protocol.	12, 19, 20
	A new SS1 solution (now only 10 µl have to be added not 15 µl as previously).	12, 19, 20
	The total volume of the second strand synthesis reaction is now 40 µl (previously it was 50 µl).	12, 19, 20
	Post second strand synthesis only 16 µl PB have to be added (previously it was 20 µl).	13, 19, 20
	In step 16 (was previously step 17) only 56 µl PS have to be added (previously it was 72 µl).	13, 19, 20
	For dual indexing the PCR buffer from the basic kit (yellow cap) has to be exchanged with the PCR buffer (purple cap) included in the i5 Dual Indexing Add-on Kit.	16, 20
	Dual indexing PCR has a final volume of 35 µl (i7 single indexing PCR has a final volume of 30 µl).	17, 20
	Post dual indexing PCR 35 µl PB added (or 31.5 µl PB for low input, low quality FFPE RNA samples).	17, 20
	Table with reference values for 500 ng input RNA from different species.	25
	Barcode 00 (BC00) in PCR Add-on Kit renamed to P7 Primer 7000.	27
	qPCR endpoint determination using only 1.7 µl template and set to 50 % FU (previously 33 %). Subtract 3 cycles from determined endpoint (EP) when using 10x as much template (17 µl in EP, 1.7 µl in qPCR).	27
	Evaluation tool to check the color balance of index subsets.	32
	015.384 includes i5 Dual Indexing Add-on Kit (four i5 indices). Information on i5 and dual indexing.	33 - 36
Mar. 24, 2014	Initial Release of QuantSeq 3′ mRNA-Seq Kit (015UG009V0100).	

18. Notes

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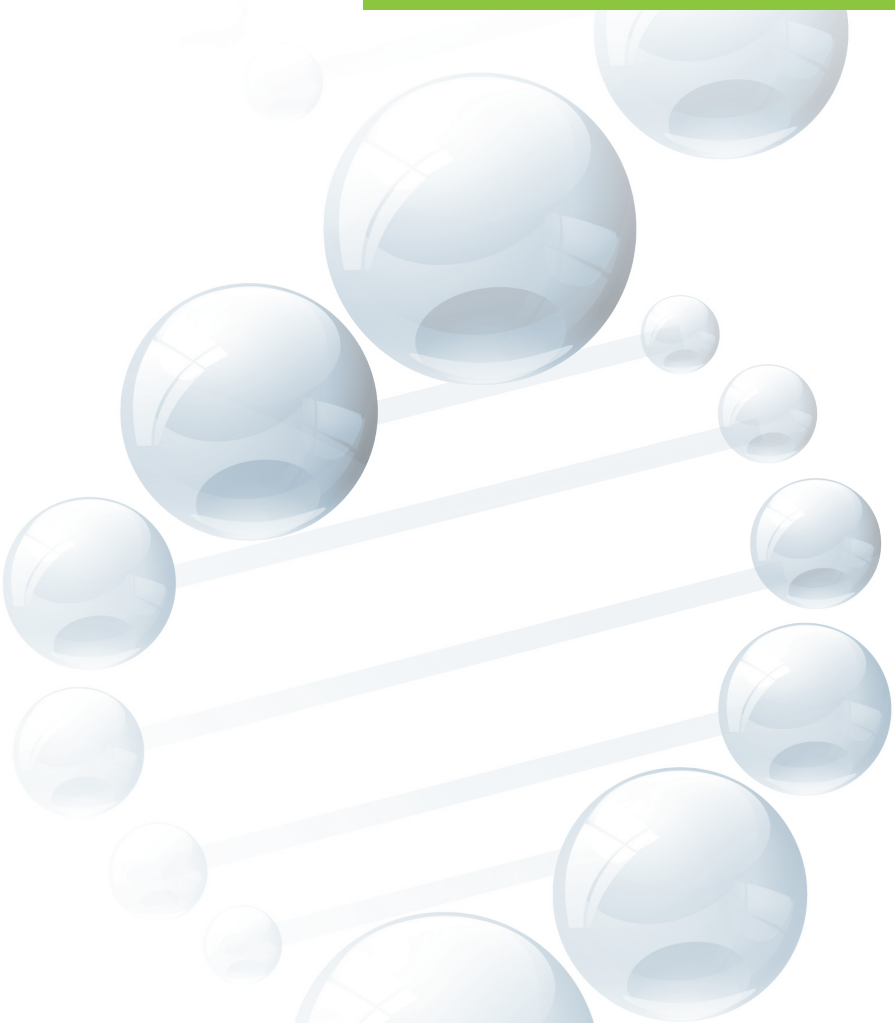
Enabling complete transcriptome sequencing



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

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