LEXOGEN

Enabling complete transcriptome sequencing

QUANT™ SEQ

Sequencing that counts

3′ mRNA-Seq Library Prep Kit FWD with Unique Dual Indices

User Guide

Catalog Numbers:
113 (QuantSeq 3′ mRNA-Seq Library Prep Kit FWD with UDI 12 nt Set A1, (UDI12A_0001-0096), 1 rxn/UDI)
114 (QuantSeq 3′ mRNA-Seq Library Prep Kit FWD with UDI 12 nt Set B1, (UDI12B_0001-0096), 1 rxn/UDI)
115 (QuantSeq 3′ mRNA-Seq Library Prep Kit FWD with UDI 12 nt Sets A1-A4, (UDI12A_0001-0384), 1 rxn/UDI)

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1. Overview

Lexogen QuantSeq 3′ mRNA-Seq Kit FWD with 12 nt Unique Dual Indices enable library preparation 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 uses total RNA as input, hence no prior poly(A) enrichment or rRNA depletion is required. Information on input requirements can be found in Appendix A, p.20. Library generation is initiated by oligodT priming (Fig. 1). The primer already contains partial Illumina-compatible linker sequence. After first strand synthesis the RNA is removed and second strand synthesis is initiated by random priming. The random primer also contains Illumina-compatible linker sequence. No purification is required between first and second strand synthesis. The insert size is optimized for shorter read lengths: SR50 - 100.

Second strand synthesis is followed by a magnetic bead-based purification step. The library is then amplified, introducing the sequences required for cluster generation. Lexogen UDI 12 nt Unique Dual Indices for multiplexing (Set A: Cat. No. 113, 115; or Set B: Cat. No. 114) are included in the kit and are introduced during the PCR amplification step. Lexogen UDI 12 nt Sets are also available as separate Unique Dual Indexing Add-on Kits (Cat. No. 107 - 111) for use with other library preps such as QuantSeq REV and QuantSeq-Flex. For these please refer to the Lexogen UDI 12 nt Unique Dual Indexing Add-on Kit Instruction Manual (107IM223).

QuantSeq Forward (FWD) 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 QuantSeq FWD. Read 2 would start with the poly(T) stretch, and sequence through the homopolymer stretch, reducing the quality of Read 2.

QuantSeq FWD maintains strand specificity to allow mapping of reads to their corresponding strand on the genome, and enabling the discovery and quantification of antisense transcripts and overlapping genes. The kits include magnetic beads for the purification steps and hence are compatible with automation.

Additional information regarding protocol modifications, PCR cycle optimization by qPCR assay, quality control, add-on modules, multiplexing, and sequencing guidelines are found in Appendices B - L (p.22 - 41). An automated QuantSeq bioinformatics pipeline has been integrated on the Bluebee® Genomics Platform and each purchased QuantSeq kit includes a code for free data analysis on this platform (see also Appendix M, p.42). For more details visit our webpage at www.lexogen.com.

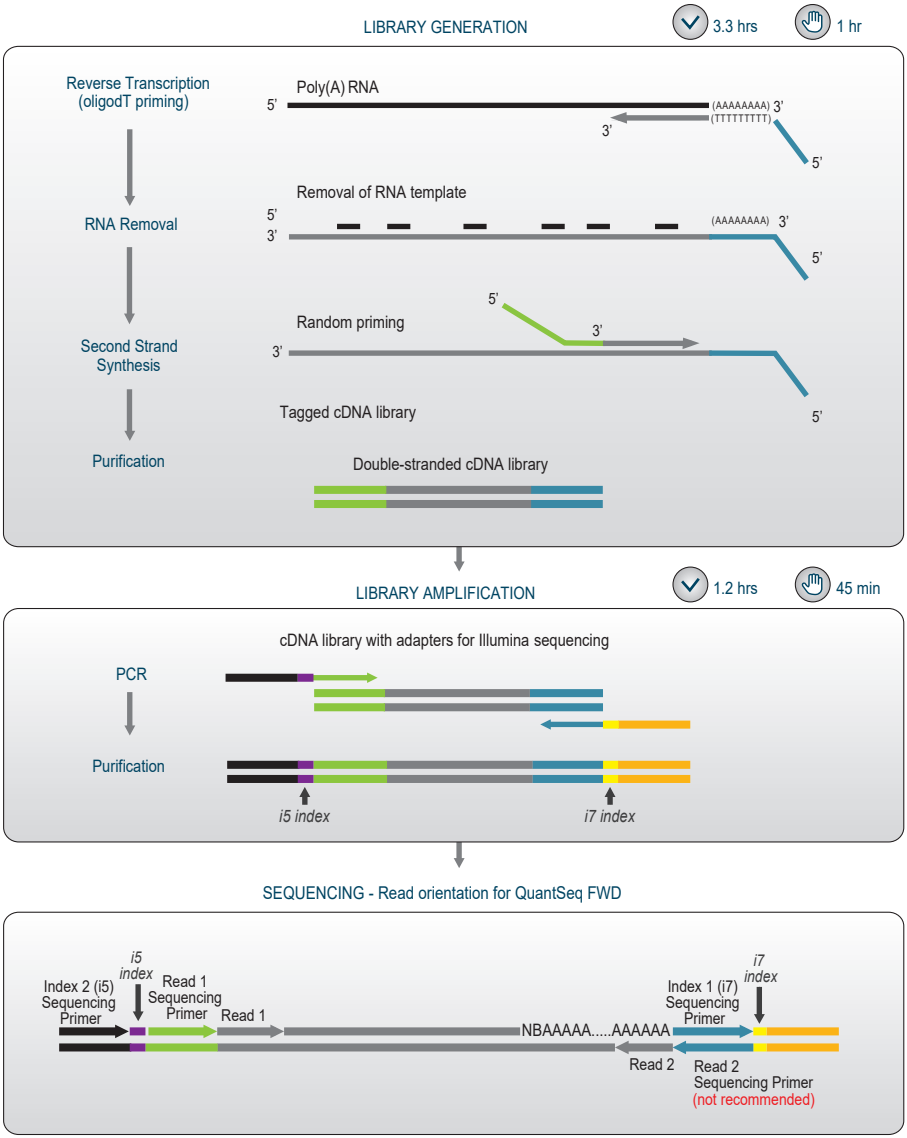


Figure 1. Schematic overview of the QuantSeq FWD library preparation workflow (Cat. No. 015, 113-115). Sequencing read orientation for QuantSeq FWD is depicted, Read 1 reflects the mRNA sequence. Paired-end sequencing is not recommended for QuantSeq FWD.

2. Kit Components and Storage Conditions

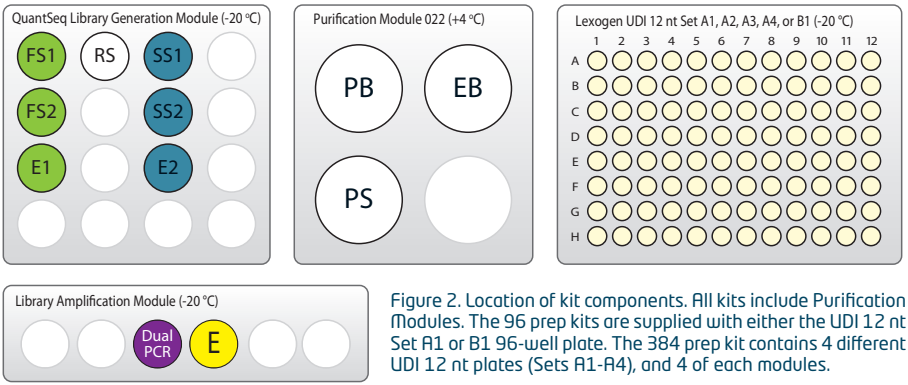


Figure 2. Location of kit components. All kits include Purification Modules. The 96 prep kits are supplied with either the UDI 12 nt Set A1 or B1 96-well plate. The 384 prep kit contains 4 different UDI 12 nt plates (Sets A1-A4), and 4 of each modules.

Kit Component	Tube Label	Volume*		Storage
		96 preps	384 preps	
First Strand cDNA Synthesis Mix 1	**FS1** ●	528 µl	4x 528 µl	-20 °C
First Strand cDNA Synthesis Mix 2	**FS2** ●	1,003.2 µl	4x 1,003.2 µl	-20 °C
Enzyme Mix 1	**E1** ●	52.8 µl	4x 52.8 µl	-20 °C
RNA Removal Solution	**RS** ○	528 µl	4x 528 µl	-20 °C
Second Strand Synthesis Mix 1	**SS1** ●	1056 µl	4x 1056 µl	-20 °C
Second Strand Synthesis Mix 2	**SS2** ●	422.4 µl	4x 422.4 µl	-20 °C
Enzyme Mix 2	**E2** ●	105.6 µl	4x 105.6 µl	-20 °C
Library Amplification Module				
Dual PCR Mix	**Dual PCR** ●	739.2 µl	4x 739.2 µl	-20 °C
Enzyme Mix	**E** ●	105.6 µl	4x 105.6 µl	-20 °C
Lexogen UDI 12 nt Sets				
Lexogen UDI 12 nt Set A1 or B1	**UDI12A/B_0001-0096**	10 µl / rxn		-20 °C
Lexogen UDI 12 nt Sets A1-A4	**UDI12A_0001-0384**		10 µl / rxn	-20 °C
Purification Module				
Purification Beads	**PB**	5,280 µl	4x 5,280 µl	+4 °C
Purification Solution	**PS**	10,772 µl	4x 10,772 µl	+4 °C
Elution Buffer	**EB**	11,616 µl	4x 11,616 µl	+4 °C / -20 °C

*including ≥10 % surplus

Upon receiving the QuantSeq kit, store the Purification Module (Cat. No. 022), containing **PB**, **PS**, and **EB** at +4 °C, and the rest of the kit in a -20 °C freezer. **REMARK: EB** can be stored either at +4 °C or -20 °C. Before use, check the contents of **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**).
- Lexogen PCR Add-on Kit for Illumina (Cat. No. 020), for qPCR assay.
- SYBR Green I (Sigma-Aldrich, Cat. No. S9430 or ThermoFisher, Cat. No. S7585), diluted to 2.5x in DMSO, for qPCR assay.

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, Inc., 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, p.20 and Appendix D, p.24, for more information on RNA quality. Consult Appendix G, 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 centrifugation 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.
- 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.
- 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.

- 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.
- To maximize reproducibility and avoid cross contamination spin down the reactions both after mixing, and after incubations at elevated temperatures (i.e., before removing the sealing foil from PCR plates or tubes, e.g., step 2).

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 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 ≥10 % surplus.

Automation

QuantSeq is compatible with automation on various platforms. For further information see Appendix N, p.44, or contact us at support@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 **or** **RS-GB** ● } thawed at RT	**SS1** ● } thawed at 37 °C **or** **USS** ● } thawed at 37 °C **SS2** ● – thawed at RT **E2** ● – keep on ice or at -20 °C	**PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – stored at +4 °C
85 °C, 3 min cool to 42 °C; 42 °C, 15 min	95 °C, 10 min cool to 25 °C	98 °C, 1 min, then cool to 25 °C (0.5 °C/sec) 25 °C, 30 min; 25 °C, 15 min	Equilibrate all reagents to room temperature for 30 minutes prior to use.

First Strand cDNA Synthesis - Reverse Transcription

An oligodT primer containing an Illumina-compatible sequence at its 5'end is hybridized to the RNA and reverse transcription is performed. To generate libraries with longer insert sizes, use the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026, Appendix H, p.30).

ATTENTION: Minimum recommended input amounts for QuantSeq FWD is 100 pg. When using input amounts ≤1ng we recommend including a no-input control (see also Appendix B, p.22).

NOTE: Protocol modifications are recommended for low input (≤10 ng), low quality, and FFPE RNA samples. These are indicated as **"REMARK"** in the respective protocol steps (see also Appendix C, p.23, and Appendix D, p.24).

1. Mix 100 pg - 500 ng of total RNA in a volume of 5 µl, with 5 µl First Strand cDNA Synthesis Mix 1 (**FS1** ●) in a PCR plate. If necessary, adjust the total volume to 10 µl with RNase-free water. Mix well by pipetting. Ensure the plate is tightly sealed, and spin down to collect the liquid at the bottom of the wells. **REMARK:** Skip this step for low input / low quality / FFPE RNA. Do not add **FS1** ● to the RNA. Place the RNA samples briefly at room temperature and proceed to step 3.

2. Denature the RNA / **FS1** mix for 3 minutes at 85 °C in a thermocycler and then cool down to 42 °C. **ATTENTION:** Leave the reactions at 42 °C until step 4. **REMARK:** Skip this step for low input / low quality / FFPE RNA.

3 Prepare a mastermix containing 9.5 µl First Strand cDNA Synthesis Mix 2 (**FS2** ●) and 0.5 µl Enzyme Mix 1 (**E1** ●) per reaction. Mix well, spin down, and pre-warm the mastermix for 2 - 3 minutes at 42 °C. **REMARK:** If step 2 is skipped, prepare a mastermix containing 5 µl First Strand cDNA Synthesis Mix 1 (**FS1** ●), 9.5 µl **FS2** ●, and 0.5 µl **E1** ● per sample. Mix well, spin down, and pre-warm for 2 - 3 minutes at 42 °C. **ATTENTION:** Do not cool mastermixes on ice.

4 Quickly spin down the denatured RNA / **FS1** samples from step 2 at room temperature to make sure all liquid is collected at the bottom of the wells. Place the samples back onto the thermocycler at 42 °C and carefully remove the sealing foil. Add 10 µl of the **FS2** / **E1** mastermix to each reaction, mix well, and seal the plate. Spin down briefly and incubate the reactions for 15 minutes at 42 °C. **REMARK:** If step 2 is skipped, add 15 µl of the pre-warmed **FS1** / **FS2** / **E1** mastermix to each 5 µl RNA sample, mix well, and seal the plate. Spin down briefly and incubate the reactions for 15 minutes at 42 °C. **OPTIONAL:** For low input / low quality / FFPE RNA, extend the incubation time to 1 hour at 42 °C. **ATTENTION:** Briefly spin down the samples and proceed immediately to step 5. Do not cool the samples below room temperature after reverse transcription.

RNA Removal

During this step the RNA template is degraded. This 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.

OPTIONAL: At step 5, the Globin Block Modules for QuantSeq (RS-Globin Block, *Homo sapiens* (**RS-GBHs** ●), Cat. No. 070; and RS-Globin Block, *Sus scrofa* (**RS-GBSs** ●), Cat. No. 071) can be used instead of the standard RNA Removal Solution (**RS** ○) (see Appendix I, p.31). The use of Removal Solution-Globin Block (**RS-GB** ●) prevents the generation of amplifiable library fragments from globin mRNAs, which are present in blood total RNA.

ATTENTION: Thaw **RS-GB** ● solutions at room temperature before use.

5 Add 5 µl RNA Removal Solution (**RS** ○) **or** 5 µl Removal Solution-Globin Block (**RS-GBHs** ● **or RS-GBSs** ●) directly to the first strand cDNA synthesis reaction. Mix well and reseal the plate using a fresh foil and spin down.

6 Incubate for 10 minutes at 95 °C, then cool down to 25 °C. Spin down and carefully remove the sealing foil. Proceed immediately to step 7. **REMARK:** Reduce the timing to 5 minutes at 95 °C for inputs ≤1 ng total RNA (see Appendix C, p.23).

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.

OPTIONAL: At step 7 the UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1, Cat. No. 081) may be used to include Unique Molecular Identifiers (UMIs) in QuantSeq FWD libraries. The UMI Second Strand Synthesis Mix (**USS** ●) replaces the Second Strand Synthesis Mix 1 (**SS1** ●) from the standard QuantSeq FWD Kit (Cat. No. 015) (see Appendix J, p.33).

ATTENTION: Important notes for Second Strand Synthesis.

- **SS1** ● and **USS** ● are viscous solutions. Thaw at 37 °C and mix thoroughly before use. If a precipitate is visible, incubate further at 37 °C, and mix until buffer components dissolve completely.
- **USS** ● is not compatible with QuantSeq REV Kits. Use only for QuantSeq FWD library preparation.

NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, and **EB**) for step 12 at room temperature to give it at least 30 minutes to equilibrate.

7	Add 10 µl Second Strand Synthesis Mix 1 (**SS1** ●) **or** 10 µl UMI Second Strand Synthesis Mix (**USS** ●) to the reaction. Mix well by pipetting, seal the plate, and spin down. **REMARK:** Use a pipette set to 30 µl for efficient mixing.
8	Incubate for 1 minute at 98 °C in a thermocycler, and slowly cool down to 25 °C at a reduced ramp speed of 0.5 °C/second. Incubate the reaction for 30 minutes at 25 °C. Quickly spin down the plate 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. **ATTENTION:** Keep the mastermix at room temperature.
10	Add 5 µl of the **SS2** / **E2** mastermix per reaction. Mix well and spin down.
11	Incubate for 15 minutes at 25 °C, then briefly spin down. ☞ 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**, and **EB**) should equilibrate for 30 minutes at room temperature before use. The Purification Beads (**PB**) must be fully resuspended before use. Thorough mixing by pipetting or vortexing is recommended.

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

12	Add 16 µl of Purification Beads (**PB**) to each reaction. Mix well, and incubate for 5 minutes at room temperature.
13	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
14	Remove and discard the clear supernatant without removing the PCR plate from the magnet. 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 fully 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. **REMARK:** For low input / low quality / FFPE RNA, add only 48 µl **PS** (see Appendix C, p.23 and Appendix D, p.24).
17	Place the plate onto a magnet 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 plate from the magnet. Do not disturb the beads.
19	Add 120 µl of 80 % EtOH, and incubate 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. 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 fully in **EB**. Incubate for 2 minutes at room temperature.
23	Place the plate onto a magnet 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. Do not transfer any beads. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

5.2. Library Amplification with 12 nt Unique Dual Indices (UDIs)

This section describes unique dual indexing PCR for multiplexing up to 384 libraries using the Lexogen 12 nt UDIs included in this kit.

Preparation

PCR				Purification (Cat. No. 022)*	
Dual PCR ●	- thawed at RT	}	**spin down before opening!**	**PB**	– stored at +4 °C
Lexogen UDI 12 nt Sets (A1 - A4, or B1)	- thawed at RT			**PS**	– stored at +4 °C
E ●	- keep on ice or at -20 °C			**80 % EtOH**	– provided by user **prepare fresh!**
				EB	– stored at +4 °C
Thermocycler	98 °C, 30 sec			Equilibrate all reagents to room temperature for 30 minutes prior to use.	
	98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec	}	11- 25x Endpoint cycle number as determined by qPCR (Cat. No. 020), see Appendix E, p.25.		
	72 °C, 1 min				
	10 °C, ∞				

PCR

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

ATTENTION: Important notes for Library Amplification.

- **Perform a qPCR assay to determine the optimal PCR cycle number for endpoint PCR**. The number of PCR cycles for library amplification must be adjusted according to RNA input amount, quality, and sample type. The PCR Add-on Kit for Illumina (Cat. No. 020) is required. For qPCR assay details see Appendix E, p.25.
- Avoid cross contamination when using the Lexogen UDI 12 nt Sets. Spin down the Index Set before opening and visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired UDIs only. Reseal opened wells with a fresh sealing foil after use to prevent cross contamination.
- Each well of the Lexogen UDI 12 nt Set is intended for single use only.

NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, and **EB**) for step 29 at room temperature to give it at least 30 minutes to equilibrate.

25 Prepare a mastermix containing 7 µl Dual PCR Mix (**Dual PCR** ●) and 1 µl Enzyme Mix (**E** ●) per reaction.

26 Add 8 µl of the **Dual PCR / E** mastermix to 17 µl of the eluted library.

27 Add 10 µl of the respective Unique Dual Index Primer pair (UDI12A_0001-0384, or UDI12B_0001-0096) to each sample. Use only one UDI per sample! **ATTENTION:** Spin down the plates containing the UDIs before opening! Pierce or cut open the sealing foil of the wells containing only the desired UDIs. Reseal opened wells of the UDI plate after use with a fresh sealing foil to prevent cross contamination!

28 Conduct 11 - 25 cycles of PCR (determine the required cycle number by qPCR) with the following program: Initial denaturation at 98 °C for 30 seconds, 11 - 25 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 Module (**PB**, **PS**, and **EB**) should equilibrate for 30 minutes at room temperature before use. The Purification Beads (**PB)** must be fully resuspended before use. Thorough mixing by pipetting or vortexing is recommended.

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

29 For **QuantSeq (standard input >10 ng)** libraries, add 35 µl of thoroughly resuspended Purification Beads (**PB**) to each reaction. **REMARK:** For **QuantSeq libraries generated from low input (≤10 ng) / low quality / FFPE RNA**, add only 31.5 µl **PB**. Mix well, and incubate for 5 minutes at room temperature.

30 Place the plate onto a magnet 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 magnet. Do not disturb the beads.

32 Add 30 µl of Elution Buffer (**EB**), remove the plate from the magnet, and resuspend the beads fully 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 magnet 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 plate from the magnet. Do not disturb the beads.

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. 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 fully in **EB**. Incubate for 2 minutes at room temperature.
40	Place the plate onto a magnet 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 G, p.28), pooling (for multiplexing, Appendix K, p.35), and cluster generation. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

6. Short Procedure

ATTENTION: Spin down before opening tubes or plates!

3.3 hrs Library Generation

Standard Input	Low Input (≤10 ng) / Low Quality / FFPE
First Strand cDNA Synthesis	
☐ Mix 5 µl RNA and 5 µl **FS1** ●.	☐ **Place RNA samples at room temp.**
☐ Incubate for 3 min at 85 °C, then cool to 42 °C. **Keep samples on thermocycler at 42 °C!**	☐ Skip!
☐ Prepare a mastermix with 9.5 µl **FS2** ● and 0.5 µl **E1** ● per reaction, mix well and pre-warm for 2 - 3 min at 42 °C.	☐ Prepare a mastermix with 5 µl **FS1** ●, 9.5 µl **FS2** ●, and 0.5 µl **E1** ● per reaction, mix well and pre-warm for 2 - 3 min at 42 °C.
☐ Add 10 µl **FS2 / E1** mix per reaction, mix well. **Keep samples on thermocycler at 42 °C when adding mastermix!**	☐ Add 15 µl **FS1 / FS2 / E1** mix per sample, mix well and spin down. **Transfer samples to thermocycler at 42 °C!**
☐ Incubate for 15 min at 42 °C. **Proceed immediately to RNA Removal!**	☐ Incubate for 15 min (**or** 1 hr) at 42 °C. **Proceed immediately to RNA Removal!**
RNA Removal	
☐ Add 5 µl **RS** ○ (**or RS-GB** ●), mix well.	☐ Add 5 µl **RS** ○ (**or RS-GB** ●), mix well.
☐ Incubate 10 min at 95 °C, cool to 25 °C.	☐ For ≤10 ng: Incubate 10 min at 95 °C; **or** for ≤1 ng: Incubate 5 min at 95 °C; then cool to 25 °C.
Second Strand Synthesis	
☐ Add 10 µl **SS1** ● (**or USS** ●), mix well.	
☐ 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.	
Purification	
☐ Add 16 µl **PB** per reaction, mix well, incubate 5 min at RT.	
☐ Place on magnet for 2 - 5 min, discard supernatant.	
☐ Add 40 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.	
☐ Add 56 µl **PS**, mix well, incubate 5 min at RT.	☐ For low input / low quality / FFPE: Add 48 µl **PS**, mix well, incubate 5 min at RT.
☐ Place on magnet for 2 - 5 min, discard supernatant.	
☐☐ Rinse 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.	

ATTENTION: Spin down before opening tubes or plates!

1.2 hrs (+qPCR) Library Amplification

Standard Input	Low Input (≤10 ng) / Low Quality / FFPE

qPCR [Strongly Recommended! Requires PCR Add-on Kit (Cat. No. 020.96)]

- ☐ Add 2 µl of **EB** to the 17 µl of eluted cDNA.
- Prepare a 2.5x stock of SYBR Green I nucleic acid stain (i.e., 1:4,000 dilution in DMSO; use Sigma-Aldrich, Cat. No. S9430 or ThermoFisher Cat. No. S7585).
- ☐ Combine 1.7 µl of cDNA with: 7 µl **PCR** ●, 5 µl Primer **7000**, 1 µl **E** ● (from PCR Add-on Kit), 1.2 µl of 2.5x SYBR Green I nucleic acid stain, and 14.1 µl of **EB**, per reaction. Mix well.
- ☐ PCR: 98 °C, 30 sec.

  98 °C, 10 sec
  65 °C, 20 sec
  72 °C, 30 sec
  } **35x** (see p.25)

  72 °C, 1 min

  10 °C, ∞. Calculate the optimal cycle number for Endpoint PCR (see Appendix E, p.25).

Endpoint PCR

- ☐ Prepare a mastermix with 7 µl Dual PCR Mix (**Dual PCR** ●) and 1 µl Enzyme Mix (**E** ●) per reaction.
- ☐ Add 8 µl of the **Dual PCR / E** mastermix to 17 µl of the eluted library.
- ☐ Add 10 µl of one Unique Dual Index Primer pair (UDI12A_0001-0384, or UDI12B_0001-0096) to each sample. **ATTENTION:** Reseal opened index wells after use! Use only one UDI/sample.
- ☐ PCR: 98 °C, 30 sec

  98 °C, 10 sec
  65 °C, 20 sec
  72 °C, 30 sec
  } **11 - 25x** (see p.25)

  72 °C, 1 min

  10 °C, ∞. ☞ Safe stopping point.

ATTENTION: Increased cycle numbers may be required for low input / low quality / FFPE RNA (see Appendix C, p.23 and Appendix D, p.24)

Purification

- ☐ Add 35 µl **PB** per reaction, mix well, incubate 5 min at RT.
- ☐ For low input / low quality / FFPE: Add 31.5 µl **PB** per reaction, mix well, incubate 5 min at RT.
- ☐ Place on magnet for 2 - 5 min, 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, 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

RNA Purity and Chemical 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). However, DNA can be removed from irreplaceable samples by acidic phenol extraction or DNase I digestion. DNase I treatment is highly recommended for FFPE RNA. 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. The DV_{200} value, which measures the percentage of RNAs larger than 200 nt in the sample, is a better measure of quality for highly degraded RNA and FFPE RNA samples with very low RIN scores.

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.

SIRV Spike-in RNA Variant Control Mixes

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

ERCC RNA Spike-in Controls

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

8. Appendix B: RNA Input and PCR Cycles

Total RNA is the intended input for QuantSeq FWD. No prior rRNA depletion or poly(A) enrichment is required. As QuantSeq is a 3' mRNA-Seq protocol both high and low quality RNA can be used as input. Any total RNA sample that contains polyadenylated mRNA can be used, including e.g., bacterial RNA samples that have been previously polyadenylated.
QuantSeq has been tested extensively high quality Universal Human Reference RNA (UHRR) across a wide range of input amounts (100 pg - 500 ng). When using input amounts ≤1 ng of total RNA, PCR cycle optimization is required and we strongly recommend including a no-input control.

Input Guidelines

- We recommend performing the protocol initially with 500 ng total RNA. RNA inputs ≥200 ng are recommended to detect low abundant transcripts efficiently.
- The minimum recommended input amounts of high-quality total RNA are 100 pg for QuantSeq FWD Library Prep.
- The minimum recommended input for QuantSeq FWD libraries prepared from whole blood total RNA, using the Globin Block Modules (RS-GB) is 50 ng. For lower input amounts, mapping rates and gene detection may be reduced.
- The maximum recommended input is 500 ng.
- Lower RNA inputs (≤10 ng), and low quality RNA samples (including FFPE) require protocol modifications, including adjusting the number of PCR cycles for the endpoint PCR (see Appendix D, p.24 and Appendix E, p.25).
- **The optimal cycle number for your specific sample type should be determined using the qPCR assay** (see Appendix E, p.25). Libraries prepared from blood total RNA with globin block, typically require one cycle more than libraries prepared from blood total RNA without globin block.
- The number of PCR cycles optimal for a given input amount of total RNA can vary by up to four and should be determined for different sample types using the qPCR assay. The table below is provided as a reference only! Optimal cycle numbers could exceed these ranges depending on the sample type (e.g., species, tissue, RNA quality (e.g., FFPE RNA).

Total RNA Input Amount	No. Cycles for Endpoint PCR**
0.5 ng*	21 - 25
10 ng*	17 - 20
100 ng	14 - 17
≥500 ng	11 - 14

* Using low input protocol modifications with 1 hour incubation at 42 °C at step 4 (See Appendix C, p.23).
** These values are provided as a **reference only!** Sample type influences the optimal cycle number, which should be determined by qPCR assay (See Appendix E, p.25).

9. Appendix C: Low Input RNA

When working with low input RNA (≤10 ng), low quality or degraded RNA, or RNA isolated from formalin-fixed, paraffin-embedded (FFPE) samples, only minor protocol modifications are recommended in order to maximize the consistency and yield of the libraries. Different protocol modifications apply when using input amounts ≤10 ng, or ≤1 ng, or when using FFPE RNA (see also Appendix D, p.24). These are outlined in the table below alongside the standard protocol steps.

<table>
<tr><th>Protocol Step</th><th>Standard Input (>10 ng)</th><th>Low Input (≤10 ng) FFPE / Degraded RNA</th><th>Low Input (≤1 ng)</th></tr>
<tr><td>Step 1</td><td>Add FS1 to RNA samples. Do not place samples back on ice after adding FS1 !</td><td colspan="2" rowspan="2">Skip denaturation step! Place RNA samples briefly at room temperature while the mastermix is prepared.</td></tr>
<tr><td>Step 2</td><td>Incubate for 3 minutes at 85 °C, then cool to 42 °C.
Hold samples at 42 °C on the thermocycler.</td></tr>
<tr><td>Step 3</td><td>Prepare FS2 / E1 mastermix – pre-warm for 2 - 3 minutes at 42 °C.</td><td colspan="2">Prepare FS1 / FS2 / E1 mastermix – pre-warm for 2 - 3 minutes at 42 °C.</td></tr>
<tr><td>Step 4</td><td>Add pre-warmed mastermix to RNA / FS1 samples on the thermocycler at 42 °C. Incubate for 15 minutes at 42 °C.</td><td colspan="2">Add pre-warmed mastermix to RNA samples at room temperature and transfer to a thermocycler preheated to 42 °C. Incubate for 15 minutes at 42 °C, or increase incubation time to 1 hour.</td></tr>
<tr><td>Step 6</td><td>Incubate for 10 minutes at 95 °C.</td><td>Incubate for 10 minutes at 95 °C.</td><td>Incubate for 5 minutes at 95 °C.</td></tr>
<tr><td>Step 16</td><td>Add 56 µl of Purification Solution (PS).</td><td colspan="2">Reduce volume of Purification Solution (PS) to 48 µl.</td></tr>
<tr><td>Step 24</td><td colspan="3">The qPCR assay is strongly recommended for optimizing the number of PCR cycles required for library amplification. This will prevent under- or overcycling of the libraries (see Appendix E, p.25): The qPCR assay should be performed also when RNA samples are of:
• Variable input amount
• Variable quality (RIN / RQN) or purity (absorbance ratios: 260/280 and 260/230)
• Variable type (e.g., species, tissue, cell type)
• FFPE origin, or highly degraded</td></tr>
<tr><td>Step 29</td><td>Add 35 µl of Purification Beads (PB) for dual-indexed libraries.</td><td colspan="2">Reduce volume of Purification Beads (PB) to 31.5 µl for dual-indexed libraries.</td></tr>
</table>

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 (see also Appendix C, p.23), specifically:

- Skipping steps 1 and 2 and preparing a mastermix of **FS1 / FS2 / E1**.
- Reducing the volume of **PS** in step 16 to 48 µl.
- Reducing the volume of **PB** in step 29 to 27 µl for single indexing PCR, and 31.5 µl in step 30 for dual indexing PCR, respectively (see FAQs at www.lexogen.com).

Further optional adjustments for low input FFPE RNA samples may also be included, such as:

- Extending the reverse transcription time in step 4 to 1 hour (≤10 ng).
- Reducing the RNA removal time in step 6 to 5 minutes 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, including 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.

ATTENTION:

- FFPE RNA samples are highly variable. Samples with lower mRNA content, or lower DV_{200} values may require more PCR cycles. **We strongly recommend performing a qPCR assay** (using Lexogen PCR Add-on Kit for Illumina (Cat. No. 020), Appendix E, p.25) to determine the optimal cycle number for library amplification.
- FFPE RNA is highly degraded, hence the insert sizes are smaller than for non-degraded RNA samples (see also Appendix G, p.28). Keep this in mind when choosing your sequencing length.
- If you see that your FFPE RNA generates ~160 bp linker-linker products despite the above-mentioned protocol changes, re-purification of the lane mix with 0.9x **PB** (e.g., 50 µl lane mix plus 45 µl of **PB**, incubating 5 minutes at room temperature, and following the protocol from step 30 on again) may be necessary.
- FFPE RNA can be contaminated with fragmented DNA, which may result in an overestimation of inserted RNA and / or in a high number intronic and intergenic reads in NGS samples. For FFPE RNA it is recommended to perform a DNase I treatment, or to distinguish between RNA and DNA when quantifying your input material. Heat inactivation of DNase I should be avoided, and the enzyme should be deactivated by other means such as phenol / chloroform extraction or silica column purification.

11. Appendix E: qPCR

Adjusting PCR Cycle Numbers for Sample Type

The mRNA content and quality of total RNA affects the number of PCR cycles needed for the final library amplification step. Variable input types and amounts require optimization of PCR cycle numbers (see Appendix B, p.22). **We strongly recommend taking advantage of the qPCR assay to optimize the number of cycles required for the endpoint PCR.** This will prevent both under and overcycling, the latter of which may bias your sequencing results (see also Appendix G, p.28).

The mRNA content of RNA samples can vary between species and tissue / cell types. Variable RNA quality, particularly for FFPE RNA samples may also affect differences in mRNA content between samples.

The PCR Add-on Kit for Illumina (Cat. No. 020) is required for the following qPCR assay protocol. This assay can be used to determine cycle numbers for subsequent dual or single indexing PCRs.

qPCR to Determine the Optimal Cycle Number for Endpoint PCR

The PCR Add-on Kit provides additional PCR Mix (**PCR** ●), Enzyme Mix (**E** ●), and the P7 Primer (**7000** ●) required for the qPCR assay. In addition, SYBR Green I nucleic acid dye (Sigma Aldrich, S9430 or ThermoFisher, Cat. No. S7585) is also needed and must be supplied by the user.

ATTENTION: Do not use the 12 nt UDIs with the PCR Mix (**PCR** ●) from the PCR Add-on Kit for Illumina (Cat. No. 020)!

NOTE: SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines has to be adjusted manually.

ATTENTION: The use of SYBR Green I-containing qPCR mastermixes from other vendors is not recommended.

1	Dilute the double-stranded library from step 24 to 19 µl by adding 2 µl Elution Buffer (**EB**) or molecular biology-grade water.
2	Prepare a 1:4,000 dilution of SYBR Green I dye in DMSO, for a 2.5x working stock concentration. **ATTENTION:** The final concentration in the reaction should be 0.1x. Higher concentrations of SYBR Green I will inhibit amplification.
3	For each reaction combine: 1.7 µl of the diluted cDNA library, 7 µl of PCR Mix (**PCR** ●), 5 µl of P7 Primer (**7000** ●), 1 µl of Enzyme Mix (**E** ●), and 1.2 µl of 2.5x SYBR Green I nucleic acid dye. Make the total reaction volume up to 30 µl by adding 14.1 µl of Elution Buffer (**EB**) or molecular biology-grade water. **ATTENTION:** Include a no template control!

4 Perform 35 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, 35 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 **REMARK:** There is no need to purify or analyze the overcycled PCR reaction on a Bioanalyzer.

5 Using the amplification curves in linear scale, determine the value at which the fluorescence reaches the plateau. Calculate 50 % of this maximum fluorescence value and determine at which cycle this value is reached. As the endpoint PCR will contain 10x more cDNA compared to the qPCR, subtract three from this cycle number. This is then the final cycle number you should use for the endpoint PCR with the remaining 17 µl of the template (see Fig. 3).

Endpoint PCR Cycle Calculation

When using 1.7 µl of cDNA for a qPCR, if the cycle number corresponding to 50 % of the maximum fluorescence is 15 cycles, the remaining 17 µl of the template should therefore be amplified with 12 cycles (15 - 3 cycles =12 cycles, Fig. 3).

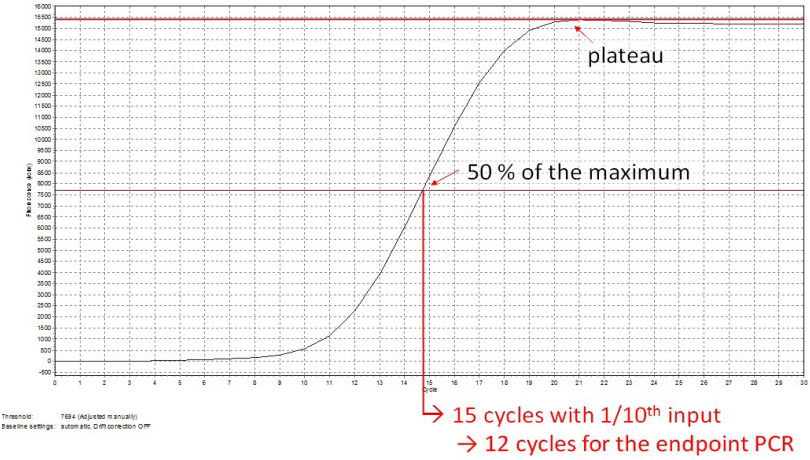


Figure 3. Calculation of the number of cycles for the endpoint PCR.

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

12. Appendix F: Library Reamplification

Reamplification of Uniquely Dual-Indexed Libraries

If your library yields are extremely low and insufficient for pooling, reamplification can be performed using the Reamplification Add-on Kit for Illumina (080.96). This kit is available only upon request. Please contact Lexogen at support@lexogen.com for more information.

Please note that the PCR Add-on Kit (Cat. No. 020) **cannot** be used for reamplification of dual-indexed libraries.

13. Appendix G: Library Quality Control

Quality control of finished QuantSeq libraries is highly recommended and should be carried out prior to pooling and sequencing. A thorough quality control procedure should include the analysis of library concentration and size distribution (i.e., library shape).

Quality Control Methods

The analysis of a small volume of the amplified library with microcapillary electrophoresis has become standard practice 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 Bioanalyzer 2100 and High Sensitivity DNA chips (Agilent Technologies, Inc.). For high throughput applications instruments such as the Fragment Analyzer or 2200 TapeStation (Agilent Technologies, Inc.), or LabChip GX II (Perkin Elmer) 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 (e.g., NanoDrop, Thermo Fisher Scientific Inc.), are not sensitive enough to accurately quantify NGS libraries at these concentrations 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. Library yield, shape, and average insert size may vary, depending on the type of input sample (e.g., FFPE samples typically produce shorter libraries than high quality Universal Human Reference RNA (UHRR), see Figures 4 and 5). The majority of inserts are greater than 75 bp in size, corresponding to final library fragment sizes ≥200 bp.

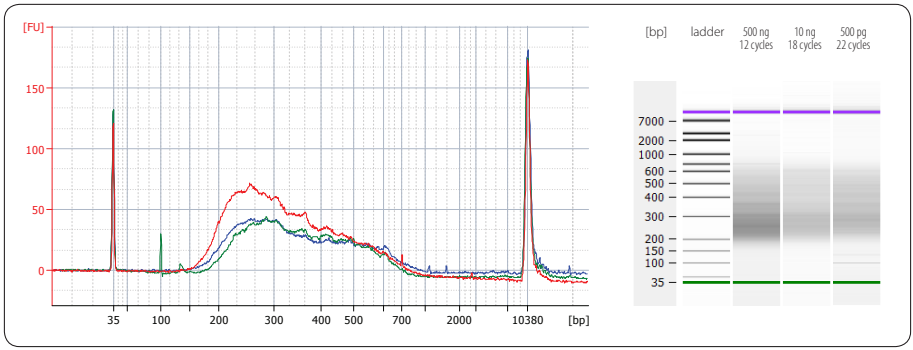


Figure 4. Bioanalyzer traces of QuantSeq FWD libraries prepared from different input amounts of total RNA input (UHRR). Libraries were prepared with the standard protocol, using 500 ng (red trace, 12 PCR cycles). Libraries with 10 ng (blue trace, 18 PCR cycles) and 500 pg (green trace, 22 PCR cycles) of UHRR input were prepared using low input protocol modifications (see Appendix C, p.23; reverse transcription for 1 hour at 42 °C for both, 5 minute incubation at 95 °C for 500 pg). Endpoint PCR was performed using the non-indexed P7 Primer 7000 (from the PCR Add-on Kit, Cat. No. 020).

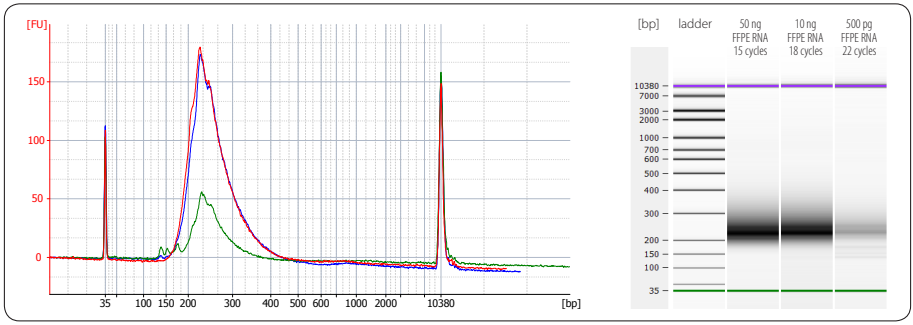


Figure 5. Bioanalyzer traces of QuantSeq FWD libraries synthesized from 50 ng (red trace), 10 ng (dark blue trace), and 500 pg (green trace), using mouse (Mm) brain FFPE RNA (RIN 1.8, DV_{200} 51 %) as input. All libraries were prepared with the recommendations for FFPE RNA input (Appendix D, p.24). 500 pg FFPE RNA libraries already contain some artifacts below 160 bp which should be removed before sequencing, e.g., by repurifying the lane mix (see Appendix K, p.35).

Overcycling

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.25.

14. Appendix H: Modulating Insert Sizes

The QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026) can be used to increase insert sizes for QuantSeq FWD libraries.

In short: **FS1** ● and **FS2** ● from the basic QuantSeq FWD kit are exchanged with **FS1x** ●, **FS2x** ●, and OligodT Primer (**dT** ●) from the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026). Longer inserts can be generated when RNA is denatured only with **dT** ● for 3 minutes at 85 °C. Longer library sizes may be beneficial for longer single-read sequencing, where increased length is beneficial for enhanced mapping rates.

For further protocol details, please see the QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2 User Guide (015UG058).

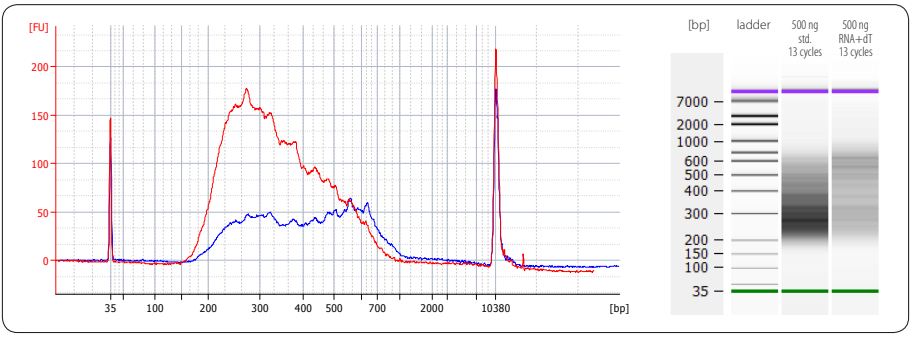


Figure 6. Bioanalyzer traces of QuantSeq FWD (std.) and Flex libraries prepared from 500 ng UHRR input RNA. Input RNA was denatured for 3 minutes at 85 °C, with either 5 µl oligodT from the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026; blue trace, RNA+dT), or the standard QuantSeq FWD FS1 buffer (red trace, std.). Average library size is increased when RNA+dT conditions are used. Libraries were amplified with dual indexing and 13 PCR cycles.

15. Appendix I: Globin Block Modules

Mammalian blood contains an abundance of globin mRNAs, which are transcribed primarily from the haemoglobin alpha and beta globin chain genes (*HBA1*, *HBA2*, and *HBB*). Lexogen's Globin Block Modules can be used with the FWD and REV QuantSeq Kits, to block the generation of library fragments from these abundant and highly stable globin mRNAs.

The Modules facilitate the depletion of globin mRNAs from blood total RNA. No prior globin depletion, poly(A) enrichment, or ribosomal RNA depletion is required. Each module consists of a modified RNA Removal Solution (RS-Globin Block, **RS-GB ●**), containing species-specific Globin Blocker oligos. The RS-Globin Block Solutions (**RS-GB ●**) simply replace the standard RNA Removal Solution (**RS ○**) at the RNA Removal step of the standard QuantSeq 3' mRNA-Seq Library Prep protocol.

The Globin Blocker oligos anneal to the 3' ends of globin first strand cDNA downstream of the random primers, and thereby prevent the generation of amplifiable library fragments from globin mRNAs during second strand synthesis (Fig. 7).

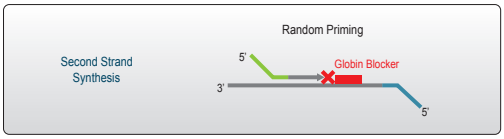


Figure 7. Globin Block Module for QuantSeq workflow.

Kit Components and Storage Conditions

Figure 8. Location of kit components.

Kit Component	Tube Label	Volume* 96 rxn	Storage
Removal Solution-Globin Block, *Homo sapiens,* 96 rxn (Cat. No. 070)	**RS-GBHs ●**	528 µl	-20 °C
Removal Solution-Globin Block, *Sus scrofa,* 96 rxn (Cat. No. 071)	**RS-GBSs ●**	528 µl	-20 °C

*including ≥10 % surplus

NOTE: RS-Globin Block, *Homo sapiens* (**RS-GBHs ●**) should be used for human blood RNA libraries. RS-Globin Block, *Sus scrofa* (**RS-GBSs ●**) should be used for pig blood RNA libraries. These Modules are designed to be species-specific. If you are interested in Globin Block for other species please contact us at support@lexogen.com.

Preparation

RNA Removal
RS-GBHs ● or RS-GBSs ● – thawed at RT
95 °C, 10 min cool down to 25 °C

Short Protocol: RNA Removal Globin Block

Removal Solution-Globin Block (**RS-GB ●**) is added at step 5 of the QuantSeq protocol and replaces the RNA Removal Solution (**RS ○**) from the standard QuantSeq 3′ mRNA-Seq Library Prep Kits (FWD: Cat. No. 015, 113 - 115, REV: Cat. No. 016).

	Follow steps 1 to 4 of the detailed protocol (p.11-12).
5	Add 5 µl of Removal Solution-Globin Block (**RS-GBHs ● or RS-GBSs ●**), directly to the first strand cDNA synthesis reaction. Mix well and reseal the plate using a fresh foil and spin down. **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.
➡	Proceed with steps 7 to 41 of the detailed protocol (p.13-17)

RNA Input and Library Amplification

The minimum recommended input for QuantSeq using Globin Block Modules is 50 ng of total RNA from whole blood, or leukocyte-enriched blood (i.e. after red blood cell lysis). Blood RNA samples may be highly variable depending on the origin and quality. The qPCR assay should be performed to determine the optimal number of cycles for library amplification (see Appendix E, p.25).

Typical Results

Example results of QuantSeq Libraries, prepared from blood RNA using the QuantSeq 3′ mRNA-Seq Library Prep Kit (FWD) and RS-Globin Block Modules are available from the on-line Frequently Asked Questions (FAQs) for QuantSeq FWD (Globin Block Specific: https://www.lexogen.com/quantseq-3mrna-sequencing/#quantseqfaq).

16. Appendix J: Unique Molecular Identifiers

Unique Molecular Identifiers (UMIs) can be included in QuantSeq FWD libraries to enable the detection and removal of PCR duplicates. The UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1) (Cat. No. 081) includes the UMI Second Strand Synthesis Mix (**USS** ●), which contains UMI-tagged random primers. The **USS** ● simply replaces the Second Strand Synthesis Mix 1 (**SS1** ●) from the standard QuantSeq FWD Kit. No other protocol changes are required. The UMIs are added between the partial P5 adapter and the random priming sequence, during second strand synthesis (Fig. 9).

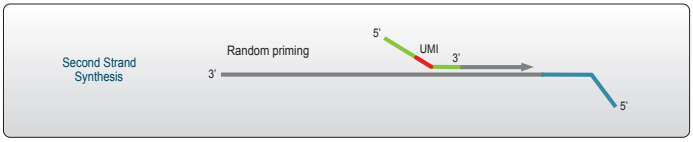


Figure 9. UMIs (red) are added during the second strand synthesis step of the QuantSeq workflow.

Kit Components and Storage Conditions

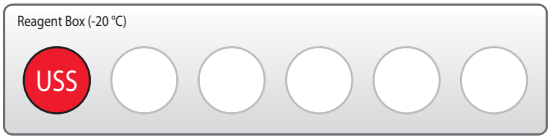


Figure 10. Location of kit component.

Kit Component	Tube Label	Volume* 96 rxn	Storage
UMI Second Strand Synthesis Mix (Cat. No. 081)	**USS** ●	1,056 µl	-20 °C

*including 10 % surplus

ATTENTION: Important notes for UMI Second Strand Synthesis Module use.

- The UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1) is not a stand-alone kit and must be used in combination with a QuantSeq FWD Kit for Illumina (Cat. No. 015, 113 - 115).
- The UMI Module is not compatible with QuantSeq REV (Cat. No. 016), or the QuantSeq 3' mRNA-Seq Library Prep Kit for Ion Torrent (Cat. No. 012).
- The UMI Second Strand Synthesis Mix (**USS** ●) replaces the Second Strand Synthesis Mix 1 (**SS1** ●) from the standard QuantSeq FWD Kit.
- The UMI Module can also be used for libraries prepared with Lexogen's Globin Block Modules for QuantSeq (Cat. No. 070, 071), and are compatible with Lexogen UDI 12 nt Unique Dual Index Sets included in this kit.

Preparation

Second Strand Synthesis
USS ● – thawed at 37 °C **SS2** ● – thawed at RT **E2** ● – keep on ice or at -20 °C
98 °C, 1 min, then cool to 25 °C (0.5 °C/sec) 25 °C, 30 min; 25 °C, 15 min

Short Protocol - Second Strand Synthesis

NOTE: This protocol replaces steps 7 and 8 of the detailed protocol (p.13). Step 8 has not been changed for UMI libraries and is included here for ease of reference.

	Follow steps 1 - 6 as indicated in the detailed protocol (p.11-13).
7	Add 10 µl of UMI Second Strand Synthesis Mix (**USS** ●) to the reaction. Mix well by pipetting, seal the plate and spin down. **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 at a reduced ramp speed of 0.5 °C/second. Incubate the reaction for 30 minutes at 25 °C. Quickly spin down the plate at room temperature before removing the sealing foil.
	Proceed to step 9 of the detailed protocol (p.13).

Sequencing*

A minimum length of 75 bp (i.e., SR75 or longer) is recommended for sequencing QuantSeq FWD libraries that include UMIs. The 6 nt UMI is read-out at the beginning of Read 1, upstream of the random priming sequence (see below). No custom sequencing primers are required.

We recommend adding a minimum of 5 - 15 % PhiX spike-in when sequencing QuantSeq FWD-UMI libraries in a pure lane-mix. For more information, please check the UMI Specific online FAQs at www.lexogen.com/quantseq-3mrna-sequencing/#quantseqfaq). Instructions for UMI data analysis are provided in Appendix M, p.42.

```
                                  5'-(Read 1 Sequencing Primer)-3'   UMI
5'AATGATACGGCGACCACCGAGATCT-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT- NNNNNN -(Insert…
3'TTACTATGCCGCTGGTGGCTCTAGA-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA- NNNNNN -(Insert…

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

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

17. Appendix K: Multiplexing

Libraries prepared with the Lexogen UDI 12 nt Unique Dual Indexing Sets are suitable for sequencing on all Illumina instruments listed below.

The Lexogen UDI 12 nt Sets enable adjustable read out of 8, 10, or the full 12 nucleotides long UDI sequence while maintaining superior error correction features. The longer the UDI read-out the higher the error correction capability.

The complete lists of i5 and i7 index sequences for all Lexogen UDI 12 nt Sets are available at www.lexogen.com/docs/indexing.

Depending on the instrument workflow and flow cell type (paired-end, PE; single-read, SR), i5 indices are sequenced differently.

Illumina Instruments	Flow Cell Type	Work-flow	Lexogen UDI 12 nt Unique Dual Indexing Kits
HiSeq 2000/2500 HiSeq 3000/4000	SR	A	Add-on Kits, **Set A1, A2, A3, and / or A4** (UDI12A_0001-0384), Cat. No. 107 – 110.96 or 120.384 QuantSeq FWD with Set A1 or A1 - A4, Cat. No. 113.96 or 115.384
HiSeq 2000/2500 MiSeq NovaSeq 6000	PE		
MiniSeq NextSeq 500/550 HiSeq 3000/4000	PE	B	Add-on **Set B1** (UDI12B_0001-0096), Cat. No. 111.96 QuantSeq FWD with Set B1, Cat. No. 114.96

Sets A1 - A4 (UDI12A_0001-0384) for Workflow A

The following Illumina instruments read the i5 index according to Workflow A:

- HiSeq 2000 / 2500 (all SR and PE flow cells)
- HiSeq 3000 / 4000 (SR flow cells only)
- MiSeq (all (PE) flow cells)
- NovaSeq 6000 (all (PE) flow cells)

For these instruments the Index 2 Read (i5) is primed using the Grafted P5 Oligo on the flow cell (or the Index 2 (i5) Sequencing Primer (HP9) for SR HiSeq 2000 / 2500 flow cells).

ATTENTION: If Lexogen UDI 12 nt Sets A1 - A4 (UDI12A_0001-0384) for Workflow A (Cat. No. 107 - 110, or 120) are used on Illumina machines with Workflow B the i5 Index will be read out as reverse complement. In this case all 12 nt of the i5 Index must be read out for error correction. Additionally, the reverse complement of the i5 index read out needs to be analyzed.

EXAMPLE: i512A_0001 is read as TTAGTAACTGGG instead of CCCAGTTACTAA. For error correction all 12 nt should be read out and the index read should be analyzed as the reverse complement in this case CCCAGTTACTAA again.

Set B1 (UDI12B_0001-0096) for Workflow B

The following Illumina instruments read the i5 index according to Workflow B:

- MiniSeq (all (PE) flow cells)
- NextSeq 500 / 550 (all (PE) flow cells)
- HiSeq 3000 / 4000 (PE flow cells only)

For these instruments the Index 2 Read (i5) is performed after Read 2 Resynthesis, using the Index 2 (i5) Sequencing Primer (see also Appendix B, p.22).

ATTENTION: If Lexogen UDI 12 nt Set B1 (UDI12B_0001-0096) for Workflow B (Cat. No. 111) is used on Illumina machines with Workflow A the i5 Index will be read out as reverse complement. In this case all 12 nt of the i5 Index must be read out for error correction. Additionally, the reverse complement of the i5 index read out needs to be analyzed.

EXAMPLE: i512B_0001 is read as TTAGTAACTGGG instead of CCCAGTTACTAA. For error correction all 12 nt should be read out and the Index read should be analyzed as the reverse complement in this case CCCAGTTACTAA again.

Index Balance

In general, it is important that each nucleotide (A, C, G, and T) is present at each position of the index reads (Index 1 Read, i7; and Index 2 Read, i5), and that the signal intensity of each nucleotide is perfectly balanced to maintain optimal base calling accuracy and read quality. This is particularly critical for instruments that use two-channel detection (e.g., NextSeq, MiniSeq, and NovaSeq), in which: T is labeled with a green fluorophore, C is labeled with red, half of A is labeled red and the other half is labeled green, and G is unlabeled.

In general, using the UDIs in numerical order as the number of libraries to multiplex increases, or column-wise for increasing multiples of 8 samples, will result in optimal nucleotide balance. For smaller numbers of samples we can also suggest the following:

- **Four libraries:** Use UDI12A / B_0001 - 0004 as these contain almost perfect nucleotide balance at each position of the index read.
- **Eight libraries:** Use column 1 of the Lexogen UDI 12 nt Set A or B (UDI12A / B_0001 - 0008).

NOTE: Individual libraries within a lane or run should always be pooled at an equimolar ratio to preserve perfect nucleotide balance at each position of the index read.

Lane Mix Preparation

Libraries should ideally be pooled in an equimolar ratio for multiplexed sequencing. It is important to ensure accurate quantification of the individual libraries prior to pooling, as well as for the library pool (lane mix). To quantify your libraries:

1. Measure the concentration of each library, using either qPCR or fluorescence-based assays (e.g., QuBit, Thermo Fisher Scientific Inc.).
2. Determine the average library size, using microcapillary electrophoresis analysis (e.g., Bioanalyzer, Agilent Technologies, Inc.). Set the range to 160 - 2,000 bp to include the whole library size distribution, and to exclude any linker-linker artifacts (160 bp), or overcycling bumps (>2,000 bp).

Molarity is then calculated from the average library size and the concentration (ng/µl) using the following equation:

$$\text{Molarity} = (\text{library concentration (ng/µl)} \times 10^6) / (660 \times \text{average library size (bp)})$$

A template for molarity calculation is also available for download from www.lexogen.com.

After pooling the libraries, the prepared lane mix and any dilutions made for denaturing (e.g., 2 nM), should be reanalyzed to ensure the accuracy of the concentration. This can be performed according to steps 1 and 2 as above.

Lane Mix Repurification to Remove Linker-Linker Artifacts

A shorter side-product representing linker-linker artifacts is sometimes visible at ~160 bp (for dual-indexed libraries), and should not compose more than 0 - 3 % of the total lane mix for sequencing. If the fraction of linker-linker, or other small fragments (≤160 bp) are too prominent, repurification of the lane mix prior to sequencing is advised.

Libraries or lane mixes can be repurified using the Purification Module with Magnetic Beads (Cat. No. 022) using the following protocol.

1. Measure the volume of the library or lane mix. If the volume is less than 20 µl, adjust the total volume to 20 µl using Elution Buffer (**EB**) or molecular biology-grade water (H_2O).
2. Add 0.9 volumes (0.9x) of Purification Beads (**PB**). Mix thoroughly and incubate for 5 minutes at room temperature. **EXAMPLE:** For 50 µl of lane mix, add 45 µl **PB.**

Follow the detailed protocol from step 30 onwards (p.16-17).

18. Appendix L: Sequencing*

The workflow for dual-indexed library sequencing differs, depending on the Illumina instrument and flow cell type. Dual indexing can be performed on single-read (SR) and paired-end (PE) flow cells. All HiSeq systems support SR and PE flow cells. NextSeq, MiniSeq, MiSeq, and NovaSeq systems use PE flow cells only, which can also be used in single-read mode. Illumina defines Workflows A and B for dual indexing read-out, which refer to the order of Index 2 read-out in relation to Read 2 Resynthesis. Lexogen defines four different workflows used for Index 2 Read (i5) read-out on Illumina instruments, which take into account also the differences in flow cell types and sequencing primers used for Index 2 (i5) read-out. These are summarized in the table below along with the compatible UDI 12 nt sets recommended for each.

<table>
<tr><th>UDI 12 nt Sets (Illumina Workflow)</th><th>Lexogen Work-flow[1]</th><th>Instrument(s)</th><th>Flow Cell Type</th><th>i5 Index Read Primer</th><th>i5 Index Read Orientation</th><th>No. of Chemis-try-Only Cycles[2]</th></tr>
<tr><td rowspan="3">A1 - A4
(A)</td><td>1</td><td>HiSeq 2000/2500</td><td rowspan="2">SR</td><td>Multiplexing Index 2 (i5) Sequencing Primer (HP9)</td><td rowspan="3">Forward (i5)</td><td>0</td></tr>
<tr><td>2</td><td>HiSeq 3000/4000</td><td rowspan="2">Grafted P5 Oligo</td><td rowspan="2">7</td></tr>
<tr><td>3</td><td>HiSeq 2000/2500
MiSeq
NovaSeq 6000</td><td rowspan="3">PE</td></tr>
<tr><td rowspan="2">B1
(B)</td><td rowspan="2">4</td><td>MiniSeq
NextSeq 500/550</td><td>Multiplexing Index 2 (i5) Sequencing Primer (HP14)</td><td rowspan="2">Reverse complement (i5rc)</td><td rowspan="2">0</td></tr>
<tr><td>HiSeq 3000/4000</td><td>Multiplexing Index 2 (i5) Se-quencing Primer (dual-index-ing primer mix)</td></tr>
</table>

[1]Workflows 3 and 4 correspond to Illumina's Dual-Indexed Workflows A and B on paired-end flow cells, respectively.
[2]Additional chemistry-only (no-imaging) cycles are performed before the i5 index is read-out.

The order of sequencing for Lexogen Workflows 1 - 3 (Illumina Workflow A) is: Read 1, index read preparation, Index 1 Read (i7), Index 2 Read (i5), Read 2 Resynthesis, and Read 2 for paired-end flow cells / runs.
The order of sequencing for Workflow 4 (Illumina Workflow B) is: Read 1, index read preparation, Index 1 Read (i7), Read 2 Resynthesis, and Index 2 Read (i5), and Read 2 for paired-end runs.

For paired-end runs, Read 2 is read-out after the Index 2 Read (i5). The order of Read 2 Resynthesis with respect to the Index 2 Read (i5) determines whether or not the i5 index is sequenced in forward or reverse complement orientation.

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

Sequencing Workflows for Dual-Indexed Libraries

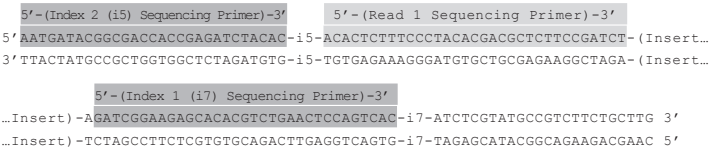
The following section depicts the library adapters and sequencing primer binding sites for QuantSeq. The sequences of the relevant Index 2 Read (i5) Sequencing Primers are also provided.

Workflow 1: Single-Read Flow Cells - HiSeq 2000 / 2500

The Index 2 (i5) Sequencing Primer (included in HP9) is required for Index 2. A minimum of eight cycles with imaging are required for i5 Index read-out, provided the correct UDI set is used for the correct i5 Index read out workflow. UDIs are 12 nt long. 12 nt, 10 nt or 8 nt can be read out optionally.

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

QuantSeq FWD libraries:



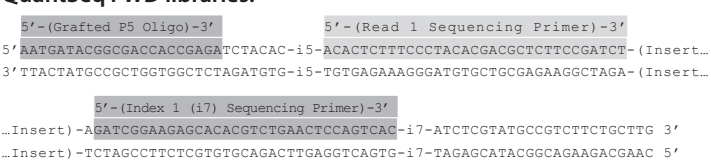
Workflow 2: Single-Read Flow Cells - HiSeq 3000 / 4000

The Grafted P5 Oligo is used to initiate index 2 (i5) read-out. Seven additional chemistry-only cycles (no imaging) plus a minimum of eight cycles with imaging are required for i5 Index read-out, provided the correct UDI set is used for the correct i5 Index read out workflow. UDIs are 12 nt long. 12 nt, 10 nt or 8 nt can be read out optionally.

Index 2 Read (i5): Grafted P5 Oligo on flow cell (not supplied):

5′ AATGATACGGCGACCACCGAGA 3′

QuantSeq FWD libraries:

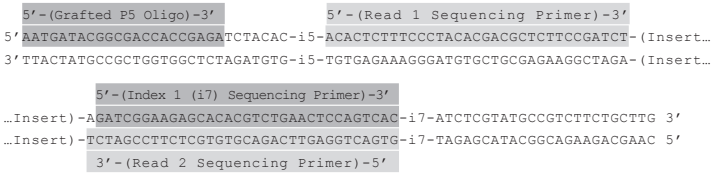


Workflow 3: Paired-End Flow Cells - HiSeq 2000 / 2500, MiSeq, and NovaSeq

The Grafted P5 Oligo is used to initiate index 2 (i5) read-out. Seven additional chemistry-only cycles (no imaging) plus a minimum of eight cycles with imaging are required for i5 Index read-out, provided the correct UDI set is used for the correct i5 Index read out workflow. UDIs are 12 nt long. 12 nt, 10 nt or 8 nt can be read out optionally.

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

QuantSeq FWD libraries:



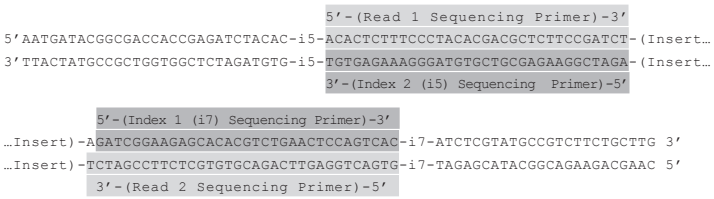
Workflow 4: Paired-End Flow Cells - MiniSeq, NextSeq, and HiSeq 3000 / 4000

All instruments use a Multiplexing Index 2 (i5) Sequencing Primer, which is included in the "Dual-Indexing Primer Mix" for MiniSeq and NextSeq, and in HP14 for HiSeq 3000 / 4000. A minimum of eight cycles with imaging are required for i5 Index read-out, provided the correct UDI set is used for the correct i5 Index read out workflow. UDIs are 12 nt long. 12 nt, 10 nt or 8 nt can be read out optionally.

ATTENTION: Index 2 (i5) is read-out after the Read 2 Resynthesis step, hence a reverse complement of the Index 2 (i5) primer sequence is produced (see also Appendix K, p.35).

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

QuantSeq FWD libraries:



Sequencing Primers

Standard Illumina sequencing primers are used for all dual-indexed libraries.
The Multiplexing Read 1 Sequencing Primer is always used for Read 1 sequencing, and the Index 1 (i7) Sequencing Primer is always used for Index 1 Read (i7) sequencing. The Index 2 Read (i5) is initiated using different sequencing primers specific to the instrument and flow cell type as outlined previously.

Read 1 for QuantSeq FWD, libraries:
Multiplexing Read 1 Sequencing Primer (not supplied):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

Multiplexing with Other Library Types

We do not recommend multiplexing Lexogen libraries with libraries from other vendors in the same sequencing lane.

Though this is possible in principle, specific optimization of index combinations, library pooling conditions, and loading amounts may be required, even for advanced users. Sequencing complex pools that include different library types at different lane shares may have unpredictable effects on sequencing run metrics, read quality, read outputs, and / or demultiplexing performance. Lexogen assumes no responsibility for the altered performance of Lexogen libraries sequenced in combination with external library types in the same lane (or run).

Due to size differences, libraries prepared with the Lexogen Small RNA-Seq Library Prep Kit (or any other small RNA library prep kit) should not be sequenced together with QuantSeq libraries. Please refer to the sequencing guidelines for each library type (library adapter details, loading amounts to use, and use of custom sequencing primers, etc.), which are provided in our library prep kit User Guides, and online Frequently Asked Questions (FAQs).

19. Appendix M: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of QuantSeq data and is kept as general as possible for integration with your standard pipeline.
QuantSeq FWD (Cat. No. 015, 113 - 115) 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.
For more detailed information please refer to www.lexogen.com/quantseq-data-analysis.

Demultiplexing

Demultiplexing can be carried out by the standard Illumina pipeline. Lexogen i7 and i5 12 nt index sequences are available for download at www.lexogen.com.

Additionally to the standard error-correction included in the Illumina pipeline, Lexogen's Error Correction Tool (available free of charge) can be used for higher accuracy in error correction. Please contact support@lexogen.com for more information.

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.

Trimming

The reads should be trimmed to remove adapter sequences, poly(A) / poly(T) sequences, and low quality nucleotides. Reads that are too short (i.e., <20 nt) or have generally low quality scores should be removed from the set.
In addition, for QuantSeq FWD libraries, as second strand synthesis is based on random priming, there is a higher proportion of mismatches over the first 12 nt of the reads. For QuantSeq FWD data we therefore recommend using an aligner that can perform soft-clipping of the read ends (e.g., STAR aligner) during alignment, or increasing the number of allowed mismatches to 14. Alternatively, trimming the first 12 nt of Read 1 can be performed prior to alignment when using a more stringent aligner (e.g., HISAT2). While trimming the read can decrease the number of reads of suitable length for alignment, the absolute number of mapping reads may increase due to the improved read quality.

Alignment

After filtering and trimming, reads can be aligned with a short read aligner to the reference genome. We recommend the use of STAR aligner for mapping **QuantSeq** data (FWD). The reads

may not land in the last exon and span a junction hence splice-aware aligners should be used. Bowtie2, BBMap, or BWA can also be used for mapping against a reference transcriptome.

Annotations and Read Counting

Mapping only the 3′ end of transcripts requires an annotation that 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.

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® Genomics Platform (for fastq(.gz) file sizes up to 1.5 GB). The activation code can be found on a card within the Library Generation Module (stored at -20 °C). Each provided code allows for the same number of data analysis pipeline runs as the number of reactions included in the library prep kit (i.e., for a 96 prep kit, 96 analysis runs can be performed). Activation codes for additional pipeline runs can also be purchased from Lexogen (Cat. No.'s 090, 091, 093, and 094). The FWD-specific pipeline is automatically encoded in the allocated activation code supplied with the respective QuantSeq Kit, ensuring that the correct pipeline parameters are used for the analysis.
Please visit www.bluebee.com/lexogen/ for more information and to access the data analysis pipelines. To login, enter the code received with the kit. For further inquiries, please contact support@lexogen.com.
Details of the technical parameters used for QuantSeq data analysis pipelines on the BlueBee platform are provided in the QuantSeq 3′ mRNA-Seq Integrated Data Analysis Pipeline on BlueBee Platform User Guide, available online from www.bluebee.com/lexogen.

QuantSeq FWD-UMI Data Analysis

Sequencing data from QuantSeq FWD libraries prepared with the UMI Second Strand Synthesis Module (Cat. No. 081), can be analyzed using the FWD-UMI QuantSeq Data Analysis pipelines available on the BlueBee® Genomics Platform. Simply use the activation code included with your QuantSeq FWD Library Prep Kit and select the respective "FWD-UMI" pipeline when setting up your data analysis run. For further information regarding the pipeline workflow please refer to the QuantSeq 3′ mRNA-Seq Integrated Data Analysis Pipeline on Bluebee Platform User Guide, available online from www.bluebee.com/lexogen.

An additional tool package (**collapse_UMI_bam**) is also available for command-line analysis and performs de-duplication of sequencing read counts for QuantSeq FWD-UMI data. To obtain a copy of the binary tool package for your specified operating system, or for further information on UMI data analysis methods, please contact support@lexogen.com.

20. Appendix N: Automation

Automating the process of library preparation has the advantage of avoiding sample tracking errors, dramatically increasing throughput, and saving hands-on time. QuantSeq is ideally suited to automation, and autoQuantSeq protocols are available for a range of liquid handling instruments, including but not limited to:

- Perkin Elmer: Sciclone® / Zephyr®
- Hamilton: Microlab STAR / STARlet / NGS STAR
- Agilent: NGS Workstation (NGS Bravo Option B)
- Beckman Coulter: Biomek FX^P, and Biomek i7
- Eppendorf: EpMotion® 5075

Instrument setups can vary widely, so if you are interested in autoQuantSeq scripts for these, or other liquid handling instruments not listed, please contact us at support@lexogen.com or check our online FAQs for more information (https://www.lexogen.com/quantseq-3mrna-sequencing/#quantseqautomation).

ATTENTION: For QuantSeq 3′ mRNA-Seq Library Prep Kit FWD with UDIs the following volume changes need to be considered for automation:

- Instead of 5 µl of i7 index primer, 10 µl UDI primer mix is added.
- During the post-PCR purification, 35 µl of **PB** are added for standard RNA inputs (>10 ng), and 31.5 µl of **PB** for low input (≤10 ng) / low quality / FFPE RNA.

The QuantSeq with UDI 12 nt kits provide sufficient reagents for autoQuantSeq library preparation, hence there is no separate kit for autoQuantSeq Library Prep.

Dummy reagents that mimic the QuantSeq reagent properties, designed to assist with the setup of autoQuantSeq protocols are available upon request. Please email support@lexogen.com for more information.

21. Appendix O: Revision History

Publication No. / Revision Date	Change	Page
113UG227V0100 Dec. 27, 2019	Initial Release of QuantSeq 3′ mRNA-Seq Library Prep Kits FWD with UDIs	

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Enabling complete transcriptome sequencing

Associated Products:

020 (PCR Add-on Kit for Illumina)
022 (Purification Module with Magnetic Beads)
025, 050, 051 (SIRVs Spike-in RNA Variant Control Mixes)
026 (QuantSeq-Flex First Strand Synthesis Module for Illumina)
028 (QuantSeq-Flex Second Strand Synthesis Module V2 for Illumina)
070 (RS-Globin Block, *Homo sapiens*)
071 (RS-Globin Block, *Sus scrofa*)
080 (Reamplification Add-on Kit for Illumina)
081 (UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1))
107 - 111 (Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits, Set A1, A2, A3, A4, or B1)
120 (Lexogen UDI 12 nt Unique Dual Indexing Add-on Kit, Sets A1-A4)

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

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