LEXOGEN

Enabling complete transcriptome sequencing

QUANT™ SEQ POOL

Sequencing that counts

Sample-Barcoded 3' mRNA-Seq Library Prep Kit

User Guide

Catalog Numbers:
139 (QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq Library Prep Kit)

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Table of Contents

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1. Overview

Lexogen's QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq Kit enables library preparation to generate Illumina-compatible libraries from polyadenylated RNA within 4.5 hours. Sample-barcodes are introduced in the first step of the protocol, during reverse transcription. Early pooling of individually labeled samples increases multiplexing capacity while tremendously reducing handling time for subsequent steps. The QuantSeq protocols generate 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. Early multiplexing and batch processing further increases reproducibility by reducing technical variability.

QuantSeq-Pool uses total RNA as input, hence no prior poly(A) enrichment or ribosomal RNA (rRNA) depletion is required. Information on input requirements can be found in Appendix A, p.21. Library generation is initiated by oligo(dT) priming (Fig. 1). The primer already contains a partial Illumina-compatible linker sequence, Unique Molecular Identifiers (UMIs), and an i1 sample-barcode. After first strand synthesis, samples are combined by pooling and purified in batch to decrease the volume for subsequent reaction steps. The RNA is removed and second strand synthesis is initiated by random priming. The random primer also contains an Illumina-compatible linker sequence. The insert size is optimized for longer read lengths using a partial paired-end setup. Library lengths are compatible with sequencing up to 500 bases.

Second strand synthesis is followed by a magnetic bead-based purification step. The library pool is then amplified, introducing the sequences required for cluster generation. At this step additional i5 / i7 indices can be added to increase the multiplexing capacity for sequencing. Without additional indexing 96 samples can be combined in one Next Generation Sequencing (NGS) run and demultiplexed according to their respective i1 sample-barcodes. For multiplexing of more than 96 samples, Unique Dual Indexing for the individual pools (containing up to 96 sample-barcoded libraries) is recommended. Lexogen offers UDI 12 nt Sets for unique dual indexing (Cat. No. 105, contact Lexogen for more options). The UDI 12 nt Sets contain pre-mixed i5 and i7 indices with superior error correction capacity for massive multiplexing. Lexogen's indices are provided in a convenient 96-well format for multiplexing of up to 9,216 samples per run for each 96-well index plate. As the i1 sample-barcode and the UMI sequence are located at the beginning of Read 2 (Fig. 1), between 18 - 22 nucleotides of Read 2 need to be sequenced to access this information.

QuantSeq-Pool 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. A poly(T) stretch corresponding to the priming site is located downstream the i1 sample-barcode and UMI sequences, therefore it is recommended to keep Read 2 to 22 nucleotides and rather use a longer Read 1 for high quality sequencing reads and to pinpoint the exact 3' end. QuantSeq-Pool further 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.

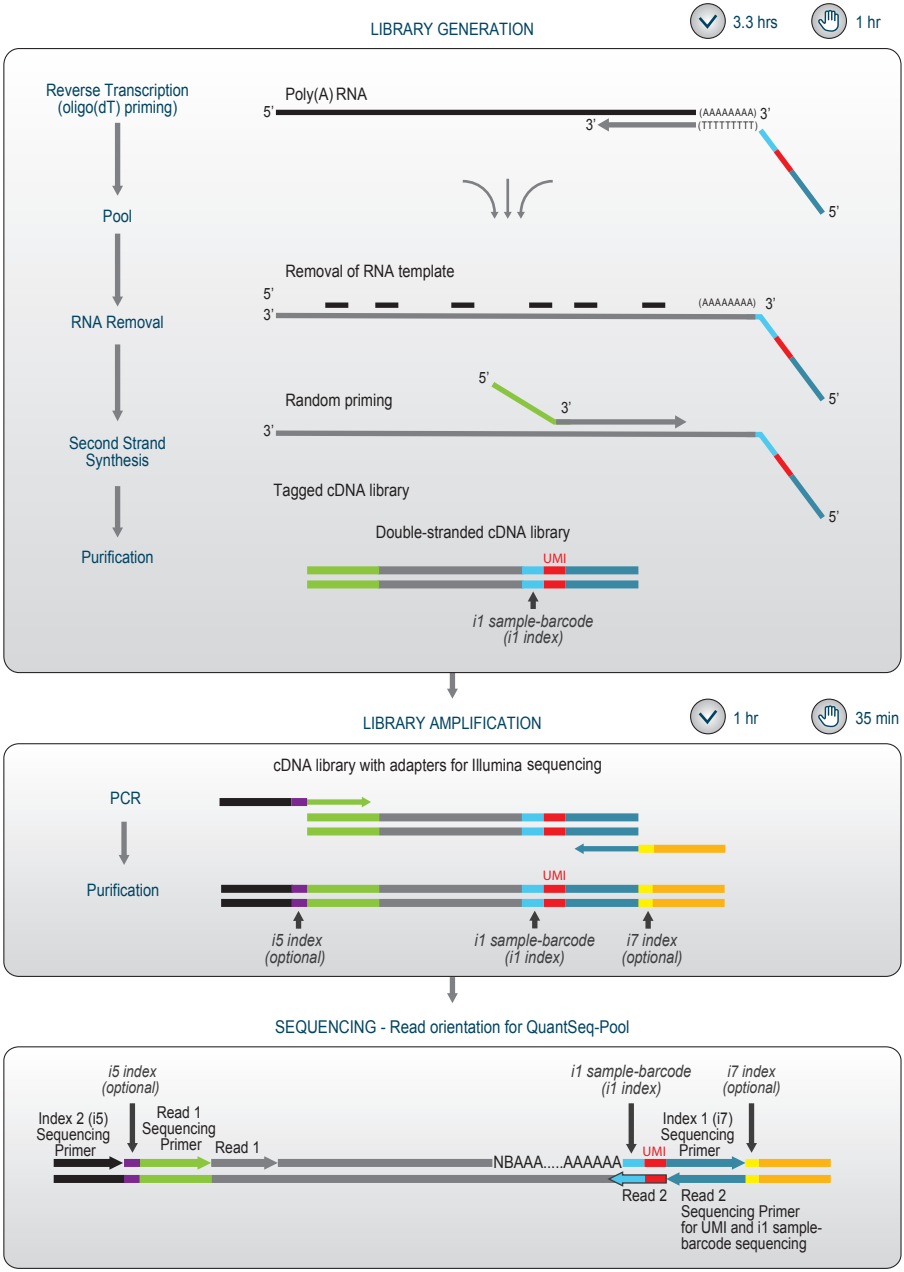

Figure 1. Schematic overview of the QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq library preparation workflow (Cat. No. 139). Sequencing read orientation for QuantSeq-Pool is depicted, Read 1 reflects the mRNA sequence. A limited paired-end run, sequencing a maximum 22 nucleotides of Read 2, is required to read out the UMI and i1 sample-barcode (i1 index) sequences. Additional i5 / i7 indices are required for multiplexing of more than 96 samples.

2. Kit Components and Storage Conditions

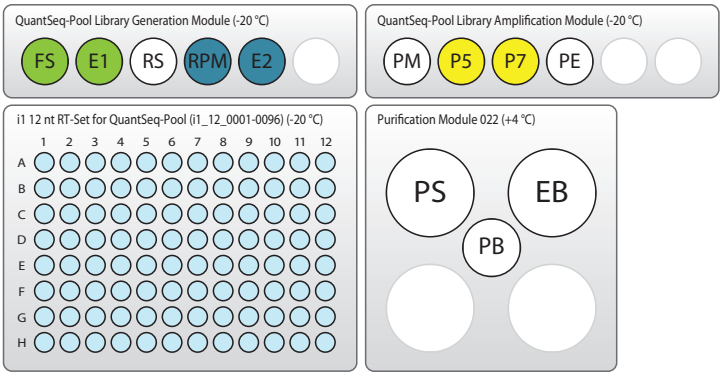

Figure 2. Location of kit components. All kits include Purification Modules. Dried-in i1 sample-barcode RT primers are provided in 96-well plates (i1 12 nt RT-Set for QuantSeq-Pool (i1_12_0001-0096)). Each kit contains a Library Generation Module and Library Amplification Module with generic P5 and P7 primers.

Kit Component	Tube Label	Volume*	Storage
i1 12 nt RT-Set for QuantSeq-Pool (96-well plate, dried-in), RTP		1 i1/ rxn	-20 °C
First Strand cDNA Synthesis	**FS** ●	290.4 µl	-20 °C
Enzyme Mix 1	**E1** ●	26.6 µl	-20 °C
RNA Removal Solution	**RS** ○	26.4 µl	-20 °C
Random Primer Mix	**RPM** ●	85.8 µl	-20 °C
Enzyme Mix 2	**E2** ●	13.2 µl	-20 °C
Library Amplification Module			
PCR Mix	**PM** ○	92.4 µl	-20 °C
P5 Primer	**P5** ●	5 µl / rxn	-20 °C
P7 Primer	**P7** ●	5 µl / rxn	-20 °C
PCR Enzyme Mix	**PE** ○	13.2 µl	-20 °C
Purification Module			
Purification Beads	**PB**	825 µl	+4 °C
Purification Solution	**PS**	2,192 µl	+4 °C
Elution Buffer	**EB**	1,598 µl	+4 °C / -20 °C

*including ≥10 % surplus

Upon receiving the QuantSeq-Pool 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. 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.21 for more information on RNA quality. Consult Appendix E, p.24 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 1 µl of enzyme in step 19 use a pipette set to 15 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, **RPM** ●, **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 step 3 and 34 of the QuantSeq-Pool 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. A minimum of 8 reactions and a maximum of 96 reactions can be processed in parallel using this kit.

EXAMPLE: Step 3 for 8 preps: use 24.2 µl **FS** ● (= 2.75 µl x 8 rxn x 1.1)
+ 2.2 µl **E1** ● (= 0.25 µl x 8 rxn x 1.1)
resulting in a total of 26.6 µl.
Step 3 for 96 preps: use 290.4 µl **FS** ● (= 2.75 µl x 96 rxn x 1.1)
+ 26.4 µl **E1** ● (= 0.25 µl x 96 rxn x 1.1)
resulting in a total of 316.8 µl.

All reagents of the QuantSeq-Pool kit include ≥ 10 % surplus.

5. Detailed Protocol

5.1 Library Generation

Preparation

First Strand cDNA Synthesis	RNA Removal	Second Strand Synthesis	Purification
RTP – thawed at RT **FS** ● – thawed at RT **E1** ● – keep on ice or at -20 °C	**RS** ○ – thawed at RT	**RPM** ● – 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, 3 min; 30 °C, 30 min	Equilibrate all reagents to room temperature for 30 minutes prior to use.

First Strand cDNA Synthesis - Reverse Transcription

An oligo(dT) primer containing an i1 sample-barcode, a Unique Molecular Identifier (UMI), and Illumina-compatible sequence at its 5′ end is hybridized to the RNA and reverse transcription is performed.

ATTENTION: Minimum recommended samples to be processed at a time is 8 reactions i.e., one column of the dried-in RT primer plate. The recommended input for an individual Reverse Transcription is 10 ng total RNA. Pooling capacity is dependent on the input amount, e.g., using 10 ng total RNA input per reaction, a minimum of 8 and a maximum of 96 reactions can be pooled into one Second Strand Synthesis reaction. When using higher input RNA amounts, a lower number of reactions can be pooled, e.g., for 120 ng total RNA input the minimum and maximum of reactions that can be pooled is 8. Ensure that the input amount per reaction is quantified accurately, variations in input RNA amounts will influence the distribution of sequencing reads between individual samples.

Input Amount per RT Reaction	Min. No. of Pooled Reactions	Max. No. of Pooled Reactions
10 ng	8 reactions	96 reactions
17 ng	8 reactions	56 reactions
20 ng	8 reactions	48 reactions
40 ng	8 reactions	24 reactions
60 ng	8 reactions	16 reactions
120 ng	8 reactions	8 reactions

NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, **and EB**) for step 5 at room temperature to give it at least 30 minutes to equilibrate.

1	Add 10 ng of total RNA in a volume of 7 µl to the desired dried-in sample-barcode RT primers provided in the 96-well PCR plate (**RTP**). Mix well to dissolve the RT primer. **ATTENTION:** Avoid cross-contamination! When processing less than 96 samples at a time, only open those wells needed for the experiment, dissolve the dried RT primer and transfer the reaction into a fresh PCR plate. Take care to avoid cross-contamination when transferring the samples!
2	Denature the RNA / **RT Primer** 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.
3	Prepare a mastermix containing 2.75 µl First Strand cDNA Synthesis Mix (**FS** ●) and 0.25 µl Enzyme Mix 1 (**E1** ●) per reaction. Mix well, spin down, and pre-warm the mastermix for 2 - 3 minutes at 42 °C. A mimimum of 8 reactions must be processed in one experiment. **ATTENTION:** Do not cool mastermixes on ice.
4	Quickly spin down the denatured RNA / **RT Primer** samples from step 2 at room temperature to make sure all liquid is collected at the bottom of the wells. Place the samples back onto the thermocycler at 42 °C and carefully remove the sealing foil. Add 3 µl of the **FS** / **E1** mastermix to each reaction, mix well, and seal the plate. Spin down briefly and incubate the reactions for 15 minutes 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.

Pooling and Purification of sample-barcoded RTs

After the RT reaction each sample contains a sample specific barcode (i1), enabling pooling and processing of all samples in one second strand synthesis reaction. The pooled RT reactions are 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.

5	Pool 9 µl of each RT reaction (a minimum of 8 RT reactions) in a 1.5 ml tube. For 8 reactions this would result in 72 µl of pooled RTs, for 48 RT reactions 432 µl, and for 96 reactions 864 µl. **ATTENTION:** For most efficient purification we recommend processing a maximum of 56 samples in 1 purification reaction. **REMARK:** For pooling of 96 reactions we recommend to split the pool into 2 parallel purification reactions for most efficient purification. Samples can later be combined again (see step 12 for details) and used in one second strand synthesis reaction.

6 Add 24 µl of Purification Beads (**PB**) and the required volume of Purification Solution (**PS**) listed in the table below to the pooled RT reactions. Mix well, and incubate for 5 minutes at room temperature.

No. of RT Reactions Pooled	Volume after Pooling	PB	PS	Volume of 80 % EtOH (step 9)
8 reactions	72 µl (8x 9 µl)	24 µl **PB**	84 µl **PS**	180 µl 80% EtOH
16 reactions	144 µl (16x 9 µl)	24 µl **PB**	192 µl **PS**	400 µl 80% EtOH
24 reactions	216 µl (24x 9 µl)	24 µl **PB**	300 µl **PS**	580 µl 80% EtOH
48 reactions	432 µl (48x 9 µl)	24 µl **PB**	624 µl **PS**	1120 µl 80% EtOH
56 reactions	504 µl (72x 9 µl)	24 µl **PB**	732 µl **PS**	1300 µl 80% EtOH
96 reactions	864 µl (96x 9 µl)	2x 24 µl **PB**	2x 624 µl **PS**	2x 1120 µl 80% EtOH

7 Place the plate (if only 8 reactions were pooled) or 1.5 ml tube onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

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

9 Add 80 % EtOH according to the volume listed in the table under step 6 and incubate for 30 seconds. Leave the plate / tube in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.

10 Repeat this washing step once for a total of two washes. Remove the supernatant completely, as traces of ethanol can inhibit subsequent PCR reactions.

11 Leave the plate / tube 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.

12 Add 11 µl of Elution Buffer (**EB**) per well / tube, remove the plate from the magnet and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature. **OPTIONAL:** If more than 56 RT reactions (e.g., 96 RT reactions) are to be processed in one Second Strand Synthesis reaction two parallel purifications are required. To combine the samples again, elute only one of the parallel purifications in steps 12 and 13. Transfer the 11 µl eluted cDNA from the first tube onto the beads of the second parallel purification and repeat step 13. The eluate collected in step 14 now contains cDNA from both purifications.

13 Place the plate / tube onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.

14 Transfer 10.5 µl of the clear supernatant into a fresh PCR plate / tube. Do not transfer any beads. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

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.

15 Add 2 µl RNA Removal Solution (**RS** ○) directly to the purified cDNA from step 14 . Mix well and reseal the plate / tube and spin down.

16 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 17

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.

ATTENTION: Important notes for second strand synthesis.

- **RPM** ● is a viscous solution. 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.

NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, and **EB**) for step 21 at room temperature to give it at least 30 minutes to equilibrate.

17 Add 6.5 µl Random Priming Mix (**RPM** ●) per reaction. Mix well by pipetting, seal the plate, and spin down. **REMARK:** Use a pipette set to 15 µl for efficient mixing.

18 Incubate for 2 minute at 98 °C in a thermocycler, and cool down to 25 °C. Incubate the reaction for 3 minutes at 25 °C. Quickly spin down the plate before removing the sealing foil.

19 Add 1 µl of **E2** per reaction. Mix well and spin down. **REMARK:** Use a pipette set to 15 µl for efficient mixing.

20 Incubate for 30 minutes at 30 °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.

21	Add 7 µl of Purification Beads (**PB**) to each reaction. Mix well, and incubate for 5 minutes at room temperature.
22	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
23	Remove and discard the clear supernatant without removing the PCR plate from the magnet. Make sure that accumulated beads are not disturbed.
24	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.
25	Add 52 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature.
26	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
27	Remove and discard the clear supernatant without removing the plate from the magnet. Do not disturb the beads.
28	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.
29	Repeat this washing step once for a total of two washes. Remove the supernatant completely, as traces of ethanol can inhibit subsequent PCR reactions.
30	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.
31	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.
32	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
33	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

Preparation

PCR			Purification (Cat. No. 022)
PM ○ **P5*** ● **P7*** ● **PE** ○	- thawed at RT - thawed at RT - thawed at RT - keep on ice or at -20 °C	**spin down before opening!**	**PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – stored at +4 °C
Thermocycler	95°C, 60 sec 95 °C, 15 sec 60 °C, 15 sec 72 °C, 60 sec 72 °C, 6 min 10 °C, ∞	9- 15x Endpoint cycle number as determined by qPCR (Cat. No. 020), see Appendix E, p.24.	Equilibrate all reagents to room temperature for 30 minutes prior to use.

* Primers P5 and P7 need to be replaced by barcoded primers containing either 6 nt i5 and i7 indices (Cat. No. 044.96, or 047.96) or 12 nt UDIs (Cat. No. 105) for multiplexing more than 96 samples.

Generic PCR primers (P5 and P7) without i5 and i7 indices are included in the kit. Without additional indexing 96 samples can be combined in one NGS run and demultiplexed according to their respective i1 sample-barcodes (i1 index sequences). The provided PCR mix is also compatible with barcoded primers that are available for purchase in addition to this kit. Lexogen offers various indexing systems that are fully compatible. For multiplexing of more than 96 samples, Lexogen offers 12 nt UDI 12 nt Sets (Cat. No.105) with pre-mixed i5 and i7 barcodes. For further information on multiplexing see Appendix F, p.29.

Between 18 and 22 nucleotides of Read 2 need to be sequenced to read out the i1 sample-barcode which is preceded by a 10 nucleotide UMI.

PCR

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

ATTENTION: Important notes for Library Amplification.

- **PCR reagents for up to 12 PCR reactions are provided with this kit.**
- **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.24.

NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, and **EB**) for step 37 at room temperature to give it at least 30 minutes to equilibrate.

34 Prepare a master mix containing 7 µl Dual PCR Mix (**PM** ○), 5 µl non-indexed PCR primer **P5** ●, 5 µl non-indexed PCR primer **P7** ● and 1 µl PCR Enzyme Mix (**PE** ○) per reaction. **OPTIONAL:** Non-indexed PCR primer **P5**, and non-indexed PCR primer **P7** may be substituted using 5 µl of the respective indexed 6 nt PCR primer available as Cat. No., 44.96, and Cat. No., 047.96, or by using 10 µl of pre-mixed i5 / i7 12 nt UDI primers available as Cat. No.105 at Lexogen. For further indexing options, please contact Lexogen.

35 Add 18 µl of the **PM / PE** / **P5** / **P7** mastermix to 17 µl of the eluted library.

36 Conduct 9 - 15 cycles of PCR (determine the required cycle number by qPCR) with the following program: Initial denaturation at 95 °C for 60 seconds, 9 - 15 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 60 seconds, and a final extension at 72 °C for 6 minutes, 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.

37 Add 31.5 µl of thoroughly resuspended Purification Beads (**PB**) to each PCR reaction. Mix well, and incubate for 5 minutes at room temperature.

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

39 Remove and discard the clear supernatant without removing the PCR plate from the magnet. Do not disturb the beads.

40 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.

41 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.

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

43 Remove and discard the clear supernatant without removing the plate from the magnet. Do not disturb the beads.

44	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.
45	Repeat this washing step once for a total of two washes. Remove the supernatant completely.
46	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.
47	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.
48	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
49	Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Do not transfer any beads. The library pools are now finished and ready for quality control (Appendix E, p.24). See Appendix F, p.26 for multiplexing of pools. ☞ 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

First Strand cDNA Synthesis - Reverse Transcription

- ☐ Add 7 µl RNA (10 - 120 ng total RNA amount) to the dried RT primer and mix well. **ATTENTION: Avoid cross-contamination (see p.12)!**
- ☐ Incubate for 3 min at 85 °C, then cool to 42 °C. **Keep samples on thermocycler at 42 °C!**
- ☐ Prepare a mastermix with 2.75 µl **FS** ● and 0.25 µl **E1** ● per reaction, mix well and pre-warm for 2 - 3 min at 42 °C.
- ☐ Add 3 µl **FS2 / E1** mix per reaction, mix well. **Spin down quickly and keep samples on thermocycler at 42 °C when adding mastermix!**
- ☐ Incubate for 15 min at 42 °C. **Do not cool, immediately proceed to Pooling and Purification!**

Pooling and Purification of Sample-Barcoded RTs

- ☐ Pool 9 µl of each RT reaction into a 1.5 ml tube. **Pool at least 8 samples and adjust the maximum number of samples pooled according to the input amount!**
- ☐ Add 24 µl of **PB** and the required volume of **PS** (see p. 13), mix well and incubate 5 min at RT
- ☐ Place on magnet for 2 - 5 min, discard supernatant.
- ☐☐ Rinse beads twice with 80 % EtOH (see p.13), 30 sec
- ☐ Air dry beads for 5 - 10 min. **ATTENTION:** Do not let the beads dry too long
- ☐ Add 11 µl **EB**, remove from magnet, mix well, incubate 2 min at RT. **OPTIONAL:** Individual purifications can be combined again at this step (see p.13)
- ☐ Place on magnet for 2 - 5 min, transfer 10.5 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point

RNA Removal

- ☐ Add 2 µl **RS** ○, mix well, and incubate 10 min at 95 °C, cool to 25 °C.

Second Strand Synthesis

- ☐ Add 6.5 µl **RPM** ●, mix well, incubate 2 min at 98 °C, cool down to 25 °C and incubate 3 min.
- ☐ Add 1 µl **E2** mix per reaction, mix well and spin down.
- ☐ Incubate 30 min at 30 °C, quickly spin down. ☞ Safe stopping point.

Purification

- ☐ Add 7 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 52 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 hr Library Amplification

Endpoint PCR

- ☐ Prepare a master mix with 7 µl PCR Mix (**PM** ○), 5 µl **P5** ●, 5 µl **P7** ●, and 1 µl Enzyme Mix (**PE** ○) per reaction. **OPTIONAL:** Replace **P5** and/or **P7** with 5 µl of 6 nt i5 and/or i7 Index Primers or with 10 µl of one Unique Dual Index (UDI) Primer pair.
- ☐ Add 18 µl of the **PM/ PE / P5 / P7** mastermix to 17 µl of the eluted library pool.
- ☐ PCR: 95 °C, 60 sec

  95 °C, 10 sec
  60 °C, 15 sec
  72 °C, 60 sec
  } **9 - 15x** (see p.23-25)

  72 °C, 6 min

  10 °C, ∞. ☞ Safe stopping point.

Purification

- ☐ Add 31.5 µl **PB** per PCR 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-Pool 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. 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-Pool specifically targets the 3' end of transcripts even RNAs with a lower RIN are suitable as input material. Formalin-Fixed, Paraffin-Embedded (FFPE) material is very heterogeneous and highly variable in terms of quality, degree of cross-linking, and accessibility of the mRNA. Thus, special care must

be taken for input normalization and even after adjustment of input amount, the number of reads per sample obtained in a sequencing experiment can vary largely as a QC of individual libraries is not possible. For processing of FFPE samples the QuantSeq 3′ mRNA-Seq Library Prep Kit for Illumina (Cat. No. 015) is recommended.

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-Pool, 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-Pool 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-Pool 3′ library preparation.

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 (Thermo Fisher Scientific Inc.). For QuantSeq-Pool 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 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 and PCR Cycles

Total RNA is the intended input for QuantSeq-Pool. No prior rRNA depletion or poly(A) enrichment is required. Any total RNA sample that contains polyadenylated mRNA can be used, including e.g., bacterial RNA samples that have been previously polyadenylated.
QuantSeq-Pool has been tested extensively high quality Universal Human Reference RNA (UHRR) using 10 ng - 120 ng total RNA.

Input Guidelines

- Accurate quantification of the RNA input and exact pipetting are required to ensure equal read depth during sequencing. Prepare RNA dilutions with care and use a 10 µl pipette for addition of the RNA to the RT primer plate. Variations in input RNA amount will influence the distribution of sequencing reads among individual samples.
- The protocol is optimized for 10 ng total RNA input per reverse transcription reaction. This input amount allows full flexibility for pooling and multiplexing. A minimum of 8 reactions and a maximum of 96 reaction can be combined after the reverse transcription step.
- The minimum recommended input amount of total RNA is 10 ng per reaction.
- A minimum of 8 reactions must be pooled after reverse transcription.
- The maximum recommended input is 120 ng per reaction. When using 120 ng total RNA input, a maximum of 8 reactions can be pooled.
- The amount of total RNA per reverse transcription reaction determines the pooling capacity. The maximum amount of cDNA corresponding to 960 ng total RNA input equivalent can be processed in one second strand synthesis reaction, refer to the table on p. 11 for details.
- Lower RNA inputs (≤ 10 ng) are not suitable for QuantSeq-Pool.
- **The optimal cycle number for your specific sample type should be determined using the qPCR assay** (see Appendix E, p.24). When pooling 12x 8 reactions, the PCR Add-on Kit (Cat. No. 020.96) is required in addition. When pooling a minimum of 16 reverse transcription reactions, the provided solutions for Library Amplification (**PM**, **P5**, **P7**, **PE**) are sufficient for qPCR and final amplification for all six library pools. It is sufficient to assess each sample type once and refer to the determined optimal cycle numbers also for future experiments.
- The number of PCR cycles for a given input amount of total RNA equivalent can vary and should be determined for different sample types using the qPCR assay (see table below).

Amount of Total RNA Equivalent per Second Strand Synthesis Reaction	No. Cycles for Endpoint PCR for UHRR
80 ng (8x 10 ng)	12 – 15
240 ng (e.g., 12x 20 ng, 24x 10 ng)	11 – 14
480 ng (e.g., 24x 20 ng, 48x 10 ng)	10 – 13
960 ng (e.g., 8x120 ng, 48x 20 ng, 96x 10 ng)	9 – 12

9. Appendix C: 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.23). **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.27).

The PCR Add-on Kit for Illumina (Cat. No. 020) is only required when pooling 12x 8 reactions. When channeling a minimum of 16 reverse transcriptase reactions into one second strand synthesis reaction, the provided solutions for Library Amplification (**PM** ○**, P5** ●, **P7** ●, and **PE** ○) are sufficient for qPCR and final amplification for all six library pools. The qPCR 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 PCR Mix (**PCR** ●), Enzyme Mix (**E** ●), and the P7 Primer (**7000** ●) required for the qPCR assay. Alternatively, PCR Mix (**PM** ○), Primer **P5** ●, primer **P7** ●, and PCR Enzyme Mix (**PE** ○) provided in this kit can be used when a minimum of at least 16 reverse transcriptase reactions are pooled. 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.

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 cDNA with 7 µl PCR Mix (**PM** ○), 5 µl **P5** ●, 5 µl **P7** ●, and 1 µl Enzyme Mix (**PE** ○), 1.2 µl of 2.5x SYBR Green I nucleic acid stain, and 14.1 µl of **EB**, per reaction. Mix well. Alternatively, mix 1.7 µl of cDNA with 7 µl of PCR Mix (**PCR** ●), 5 µl of P7 Primer (**7000** ●), 1 µl of Enzyme Mix (**E** ●), 1.2 µl of 2.5x SYBR Green I nucleic acid dye and 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 95 °C for 60 seconds, 35 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 60 seconds, and a final extension at 72 °C for 6 minutes, 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).

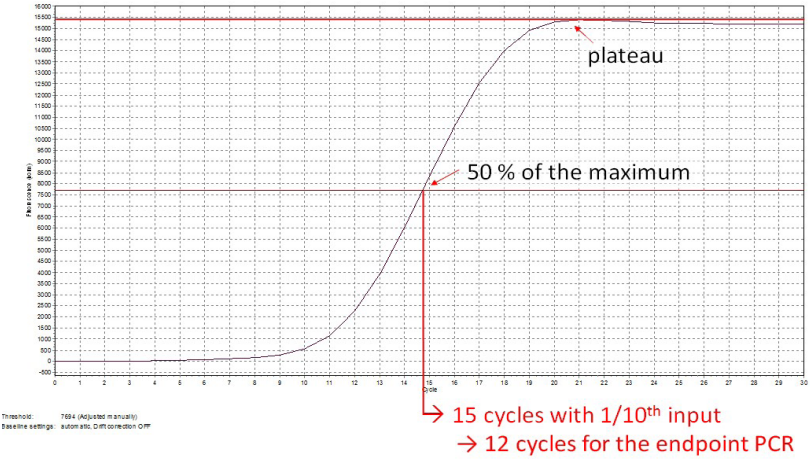

Figure 3. Calculation of the number of cycles for the endpoint PCR.

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.

10. Appendix D: Library Reamplification

Reamplification of QuantSeq-Pool 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 triple-indexed libraries.

11. Appendix E: Library Quality Control

Quality control of finished QuantSeq-Pool library pools is highly recommended and should be carried out prior to sequencing. For multiplexing of 96 sample-barcoded QuantSeq-Pool libraries the final purification after PCR amplification of the pooled libraries already yields the final lane mix. Therefore, quality control to determine the concentration and size distribution (i.e., library shape) of the lane mix is required for optimal loading onto the sequencer. Optionally, for multiplexing more than 96 QuantSeq-Pool libraries, individual pool of 8 - 96 libraries that contain additional i5 / i7 indices can be pooled in an equimolar ratio to obtain the final lane mix for sequencing. In this case, quality control should be used to determine the correct pooling ratios, see Appendix F, p.26 for more information.

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-Pool libraries 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 pools may be diluted to the required volume (e.g., 2 µl sample for TapeStation and 10 µl for LabChip GX II).

More accurate 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 pool on a polyacrylamide or agarose gel. Library pool 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-Pool 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., samples with lower RIN may produce shorter libraries than high quality Universal Human Reference RNA (UHRR)). The majority of inserts are longer than ~430 bp in size, corresponding to final library fragment sizes ≥ 600 bp.

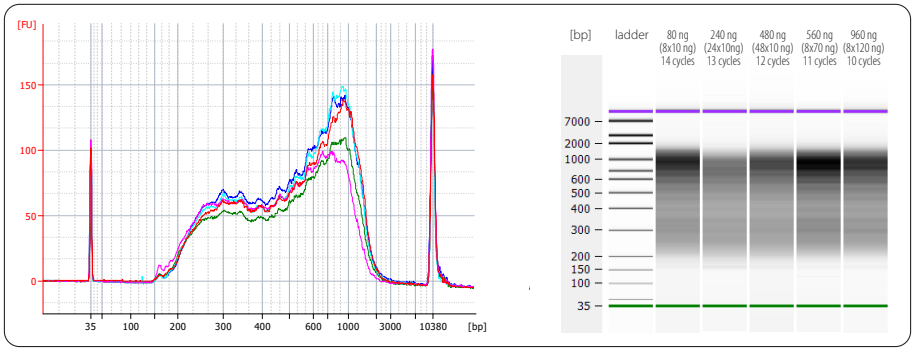

Figure 4. Bioanalyzer traces of QuantSeq-Pool libraries prepared from different input amounts of total RNA input (UHRR) and pooling various amounts of total RNA equivalents after reverse transcription. Libraries were prepared following the protocol described in this user guide pooling either 8 x 10 ng (red trace, 14 PCR cycles), 24 x 10 ng (pink trace, 13 PCR cycles), 48 x 10 ng (green trace, 12 PCR cycles), 8 x 70 ng (cyan trace, 11 PCR cycles) or 8 x 120 ng (blue trace, 10 PCR cycles) at step 5. RNA removal, second strand synthesis and library pool amplification was performed using 80 ng, 240 ng, 480 ng, 560 ng or 960 ng total RNA equivalent, respectively. Endpoint PCR was performed using 6 nt i5 / i7 dual indexing (Cat. No. 047.96 and Cat. No. 044.96) together with **PM** and **PE** provided in the QuantSeq-Pool Sample-barcoded 3' mRNA-Seq Library Prep Kit.

Overcycling

An elevated base line that extends past the upper marker is an indication of overcycling. This could occur if cycle numbers are increased too much to compensate for lower quality input material. Prevent overcycling by using the qPCR assay as described in Appendix E, p.24. Overcycled PCRs may still be used for subsequent sequencing. However, gene expression values may be biased. To guarantee accurate quantification of overcycled libraries for lane mixing, we recommend performing a qPCR-based quantification method rather than relying on the Bioanalyzer quantification. For further experiments using the same input RNA, please adjust your cycle number accordingly.

12. Appendix F: Multiplexing

Generic PCR primers (P5 and P7) without i5 and i7 indices are included in the kit. Without additional indexing up to 96 samples can be combined in one NGS run and demultiplexed according to their respective i1 sample-barcode indices.

Single Indexing - i1 sample-barcodes (i1 indices)

i1 sample-barcodes allowing up to 96 samples to be sequenced per lane on an Illumina flow cell are included in the kit (i1 12 nt RT-Set for QuantSeq-Pool, 96-well plate). i1 sample-barcodes are 12 nucleotides long and contain a nested design allowing for different index read out lengths. The i1 supports read out of either 8, 10, or 12 nucleotides depending on the multiplexing requirement of the experiment. i1 sample-barcodes are preceded by a 10 nucleotide UMI and are read out at the beginning of Read 2 using between 18 and 22 cycles for Read 2. The location of the respective i1 sample-barcodes within the 96-well plate is shown below. i1 sample-barcodes are color-balanced in numerical order as well as column-wise by increasing multiples of 8 samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	i112 0001	i112 0009	i112 0017	i112 0025	i112 0033	i112 0041	i112 0049	i112 0057	i112 0065	i112 0073	i112 0081	i112 0089
B	i112 0002	i112 0010	i112 0018	i112 0026	i112 0034	i112 0042	i112 0050	i112 0058	i112 0066	i112 0074	i112 0082	i112 0090
C	i112 0003	i112 0011	i112 0019	i112 0027	i112 0035	i112 0043	i112 0051	i112 0059	i112 0067	i112 0075	i112 0083	i112 0091
D	i112 0004	i112 0012	i112 0020	i112 0028	i112 0036	i112 0044	i112 0052	i112 0060	i112 0068	i112 0076	i112 0084	i112 0092
E	i112 0005	i112 0013	i112 0021	i112 0029	i112 0037	i112 0045	i112 0053	i112 0061	i112 0069	i112 0077	i112 0085	i112 0093
F	i112 0006	i112 0014	i112 0022	i112 0030	i112 0038	i112 0046	i112 0054	i112 0062	i112 0070	i112 0078	i112 0086	i112 0094
G	i112 0007	i112 0015	i112 0023	i112 0031	i112 0039	i112 0047	i112 0055	i112 0063	i112 0071	i112 0079	i112 0087	i112 0095
H	i112 0008	i112 0016	i112 0024	i112 0032	i112 0040	i112 0048	i112 0056	i112 0064	i112 0072	i112 0080	i112 0088	i112 0096

The Lexogen i1 sequences are available for download at www.lexogen.com/docs/indexing.

The provided PCR mix is also compatible with index primers that are available for purchase in addition to this Kit. Lexogen offers various indexing systems that are fully compatible. For multiplexing of more than 96 samples, unique dual indexing for the individual pools (containing up to 96 sample-barcoded libraries) is recommended. In this way, multiple pools can be sequenced in one run increasing the multiplexing capacity tremendously. The resulting libraries are thus triple indexed with unique i5 and i7 indexes to distinguish the different pools and i1 sample-barcodes to differentiate between individual samples within one pool. Between 18 and 22 nucleotides of Read 2 need to be sequenced to read out the i1 sample-barcode.

Lexogen UDI 12 nt Sets (Cat. No. 105) with pre-mixed i5 and i7 indices are available and offer superior error correction capacity for massive multiplexing. Lexogen's indices are provided in

a convenient 96-well format for multiplexing of up to 9,216 samples per lane for each 96-well index plate. Lexogen UDI 12 nt Unique Dual Indexing Sets are available for up to 384 12 nt UDIs increasing the multiplexing capacity to 36,864 samples per sequencing lane. Lexogen also offers 6 nt single indexing sets for i7 (Cat. No. 044.96) and i5 (Cat. No. 047.96). These 6 nt Index Sets can be used separately as single indexes for either i5 or i7, or can be combined for dual indexing.

For more information on indexing, please refer to the respective Instruction Manuals (047IM109 for 6 nt index sets and 107IM223 for 12 nt index sets).

The complete lists of i1, i5, and i7 index sequences for all Lexogen index Sets are available at www.lexogen.com/docs/indexing.

Lane Mix Preparation

Library pools should ideally be combined in an equimolar ratio for multiplexed sequencing. It is important to ensure accurate quantification of the individual library pools prior to mixing, as well as for the final lane mix. To quantify your QuantSeq-Pool libraries:

1. Measure the concentration of each library pool, using either qPCR or fluorescence-based assays (e.g., QuBit, Thermo Fisher Scientific Inc.).
2. Determine the average library pool size, using microcapillary electrophoresis analysis (e.g., Bioanalyzer, Agilent Technologies, Inc.). Set the range to include the whole size distribution, and exclude any linker-linker (LL) artifacts. For generic P5 / P7 primers this would be 170 bp - 9000 bp (L.L peak at ~150 bp). For 6 nt single indexing (i7) select 175 - 9000 bp (LL peak at ~155 bp), for 6 nt dual indexing select 185 - 9000 bp (LL peak at ~165 bp), and for 12 nt unique dual indexing use 195 - 9000 bp (LL peak at ~177 bp). An elevated base line that extends past the upper marker is an indication of over-cycling, and quantification will be biased.

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

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

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

After combining the library pools, 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 below ~195 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 (≤195 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 38 onwards (p.16-17).

13. Appendix G: Sequencing*

General

The amount of libraries loaded onto the flow cell will greatly influence the number of clusters generated. QuantSeq-Pool libraries can be sequenced using the standard Illumina Multiplexing Read 1 and Read 2 Sequencing Primers. Read 1 directly corresponds to the mRNA sequence, Read 2 is required for UMI and sample-barcode read out, therefore at least 18 to 22 nucleotides must be read out to identify the individual sample within the library pool. A schematic representation of those libraries is shown below.

Unique Molecular Identifiers (UMIs) are contained at the start of Read 2 preceding the i1 sample-barcode (see below). As QuantSeq-Pool library generation is initiated by oligo(dT) priming a polyT sequence will be present after the i1 sample-barcode sequence. Therefore, paired-end sequencing with a limited Read 2 (18 to 22 cycles) is required for QuantSeq-Pool libraries.

QuantSeq-Pool Libraries with i5 / i7 Dual Indexing

i5 and i7 indices can be introduced during PCR (step 34). For compatible i5 / i7 indices please see Appendix F, p.26. The Index 1 (i7) Sequencing Primer is always used for Index 1 Read (i7) sequencing. Index Read 2 sequencing is exemplified for the Multiplexing Index 2 (i5) Sequencing Primer. Alternative sequencing workflows may use the Grafted P5 Oligo (not shown).

```
   5'-(Index 2 (i5) Sequencing Primer)-3'      5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT- (Insert…
3'TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA- (Insert…


                        UMI   5'-(Index 1 (i7) Sequencing Primer)-3'
…Insert)-i1(12 nt)-N(10) AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
…Insert)-i1(12 nt)-N(10) TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
                          3'-(Read 2 Sequencing Primer)-5'
```

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

Read 2 for QuantSeq-Pool libraries (required for UMI and sample-barcode read out):
Multiplexing Read 2 Sequencing Primer (not supplied):
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

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

Index reads are optional. When multiplexing more than 96 samples per lane, additional i5 / i7 indices are required and need to be read out in order to distinguish the different pools.

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

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

NOTE: QuantSeq-Pool libraries are oligo(dT)-primed. Therefore, the poly(T) stretch is located at the beginning of the insert following the UMI and i1 index.

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-Pool 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).

14. Appendix H: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of QuantSeq-Pool data and is kept as general as possible for integration with your standard pipeline.
QuantSeq-Pool libraries contain the Read 1 linker sequence in the 5' part of the second strand synthesis primer, hence NGS reads are generated towards the poly(A) tail. 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 i5 / i7

Demultiplexing of i5 / i7 indices can be carried out by the standard Illumina pipeline. Lexogen i7 and i5 index sequences for 6 nt and 12 nt index systems 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) for the UDI 12 nt Unique Dual Indexing system can be used for higher accuracy in error correction. Please contact support@lexogen.com for more information.

Demultiplexing i1

The i1 sample-barcode is located at the beginning of Read 2 and preceded by a 10 nt UMI sequence (for UMI processing see p. 35). Therefore, the i1 barcode is contained within bases at position 11 - 22 of Read 2, depending on the chosen index read out length of 8, 10 or 12 nucleotides. Demultiplexing of i1 sample-barcoded libraries can be performed using Lexogen's Demultiplexing Tool (available free of charge at https://github.com/Lexogen-Tools/idemux). 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-Pool 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-Pool 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- Pool 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.

QuantSeq-Pool UMI Data Analysis

QuantSeq-Pool libraries contain 10 nt Unique Molecular Identifiers (UMI) at the beginning of Read 2. In order to analyze the UMI information, the first 10 nucleotides of Read 2 can be extracted and used to collapse the reads with well established open source tools for UMI deduplication, e.g., umi_tools.

Additionally, a tool package (**collapse_UMI_bam**) is also available for command-line analysis and performs de-duplication of sequencing read counts for QuantSeq-Pool 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.

15. Appendix I: Revision History

Publication No. / Revision Date	Change	Page
139UG272V0100 Aug. 10, 2020	Initial Release.	

LEXOGEN

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)
044 (Lexogen i7 6 nt Index Set (7001-7096), 1 rxn/Index)
047 (Lexogen i5 6 nt Unique Dual Indexing Add-on Kit (5001-5096), 1 rxn/Index)
080 (Reamplification Add-on Kit for Illumina)
105 (Lexogen UDI 12 nt Set B1)

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

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