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Sequencing that counts

Sample-Barcoded 3' mRNA-Seq
Library Prep Kit

User Guide

Catalog Number:

139 (QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq Library Prep Kit for Illumina)

139UG272V0300

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For any publication using this product, please refer to it as Lexogen's QuantSeq™-Pool Sample-Barcoded 3' mRNA-Seq Library Prep Kit for Illumina.

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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 transcripts. Early multiplexing and batch processing further increase 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.18. 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. 101 - 105, 156). 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. It is therefore 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, enabling the discovery and quantification of antisense transcripts and overlapping genes.

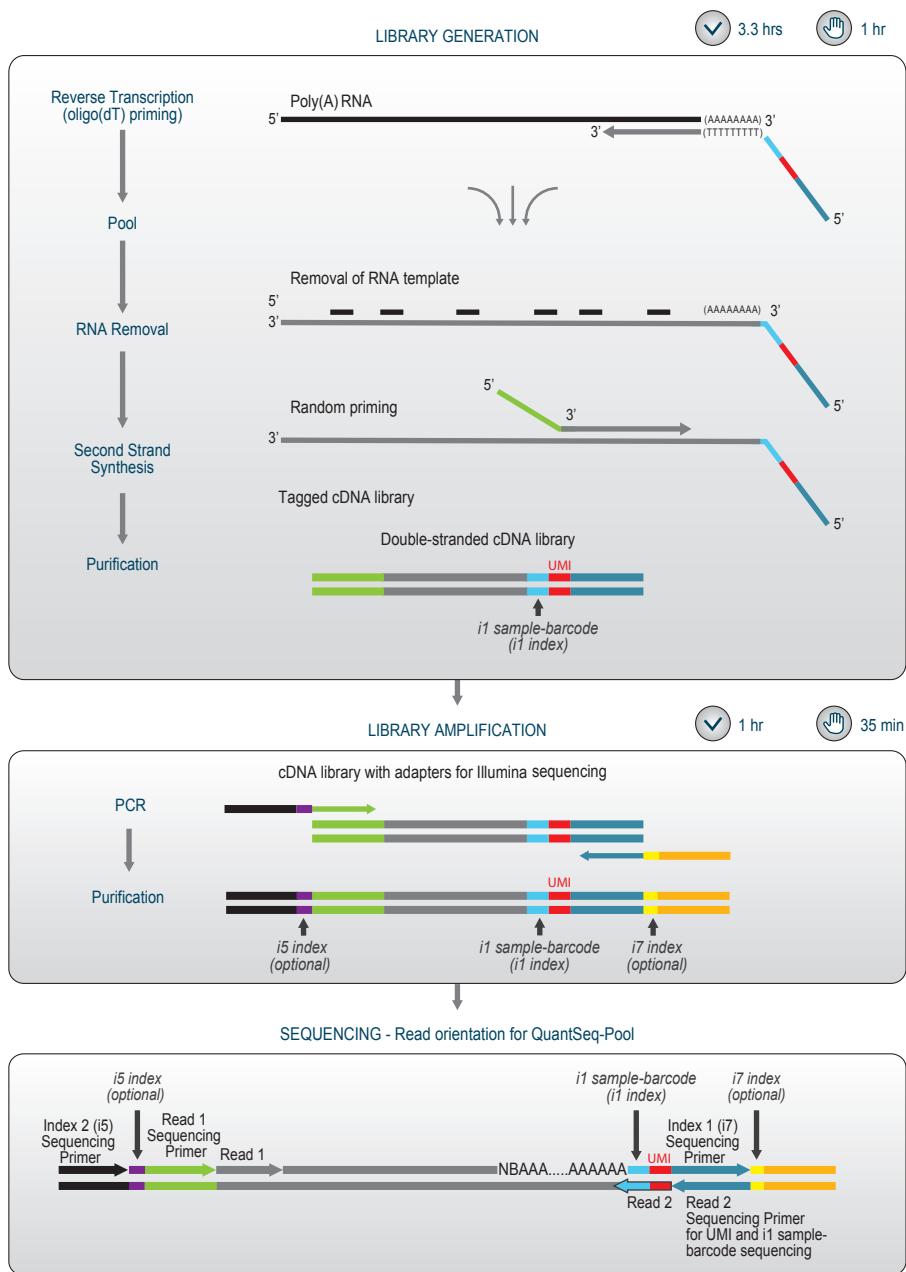


Figure 1. Schematic overview of the QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq library preparation workflow (Cat. No. 139). Sequencing read orientation for QuantSeq-Pool is depicted, Read 1 reflects the mRNA sequence. A limited paired-end run, sequencing a maximum 22 nucleotides of Read 2, is required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

2. Kit Components and Storage Conditions

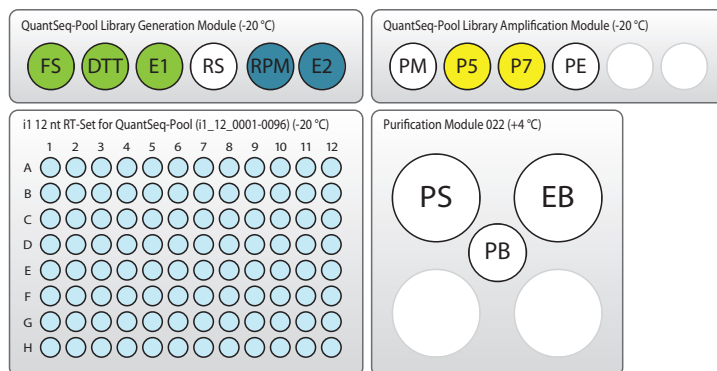


Figure 2. Location of kit components. All kits include Purification Modules. Dried-in i1 sample-barcode RT primers are provided in 96-well plates (i1 12 nt RT-Set for QuantSeq-Pool (i1_12_0001-0096)). Each kit contains a Library Generation Module and Library Amplification Module with generic P5 and P7 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 Mix	FS ●	264.0 µl	-20 °C
DTT	DTT ●	26.4 µl	-20 °C
Enzyme Mix 1	E1 ●	26.4 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 only.
- 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.
- Magnetic rack for handling 1.5 ml reaction tubes.
- 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 D, p.24 for information on library quantification methods.

4. Detailed Protocol

4.1 Library Generation

Preparation

First Strand cDNA Synthesis	RNA Removal	Second Strand Synthesis	Purification
RTP – thawed at RT FS – thawed at RT DTT – 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 25 °C; 42 °C, 15 min	95 °C, 10 min cool to 25 °C	98 °C, 2 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 an Illumina-compatible sequence at its 5' end is hybridized to the RNA and reverse transcription (RT) is performed.

ATTENTION: The minimum number of samples to be processed and pooled at a time is 8 i.e., one column of the dried-in RT primer plate. The recommended input for an individual sample is 10 ng total RNA. Pooling capacity is dependent on the input amount, e.g., using 10 ng total RNA input per sample, where a minimum of 8 and a maximum of 96 samples can be pooled into one Second Strand Synthesis reaction. When using higher input RNA amounts, less samples can be pooled, e.g., for 120 ng total RNA input, the minimum and maximum number of samples that can be pooled is 8. Ensure that the input amount per sample is quantified accurately, as variations in input RNA amounts will influence the distribution of sequencing reads between individual samples. To determine input amounts based on the number of individual samples you have, please utilize our [QuantSeq-Pool Calculator](#).

Input Amount per Sample	Min. No. of Samples Pooled	Max. No. of Samples Pooled
10 ng	8 samples	96 samples
17 ng	8 samples	56 samples
20 ng	8 samples	48 samples
40 ng	8 samples	24 samples
60 ng	8 samples	16 samples
120 ng	8 samples	8 samples

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 - 120 ng of total RNA in a volume of 7 µl to the desired dried-in sample-barcode RT primer provided in the 96-well PCR plate (**RTP**). Mix well to dissolve the RT primer. A minimum of 8 samples must be processed in one experiment. **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 25 °C. Spin down the denatured RNA / **RT Primer** reactions to make sure all liquid is collected at the bottom of the wells.

3 Prepare a mastermix containing 2.5 µl First Strand cDNA Synthesis Mix (**FS** ●), and 0.25 µl DTT (**DTT** ●) and 0.25 µl Enzyme Mix 1 (**E1** ●) per RT reaction. Mix well and spin down. **TIP:** Aliquot the mastermix into an 8-tube PCR strip, for convenient addition of mastermix to the RNA / **RT Primer** plate using a multichannel pipette.

4 Carefully remove the sealing foil from the denatured RNA / **RT Primer** plate. Use a multi-channel pipette to add 3 µl of the **FS** / **DTT** / **E1** mastermix to each RT reaction, seal the plate, and gently vortex to mix. Spin down briefly and incubate the reactions for 15 minutes at 42 °C. **ATTENTION:** Briefly spin down the reactions and proceed immediately to step 5. Do not cool the reactions below room temperature after reverse transcription.

Pooling and Purification of Sample-Barcoded RTs

After reverse transcription, each reaction 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.

ATTENTION: When pooling more than 56 RT reactions, we recommend splitting the pool and processing two parallel purification reactions. This ensures optimal purification efficiency. The reactions are then recombined during elution (see step 12) and processed as one second strand synthesis reaction.

NOTE: Before starting the purification, determine the amounts of Purification Solution (**PS**) and 80 % EtOH needed according to the number of RT reactions pooled, by using the table below or our online [QuantSeq-Pool Calculator](#). Exact formulas for calculating **PS** and 80% EtOH volumes are also provided in Appendix A, p.18.

No. of RT Reactions Pooled	Volume after Pooling	Volume of PB to add	Volume of PS to add	Volume of 80 % EtOH (step 9)
8 reactions	72 µl (8x 9 µl)	24 µl	84 µl	180 µl
16 reactions	144 µl (16x 9 µl)		192 µl	400 µl
24 reactions	216 µl (24x 9 µl)		300 µl	580 µl
48 reactions	432 µl (48x 9 µl)		624 µl	1,120 µl
56 reactions	504 µl (56x 9 µl)		732 µl	1,300 µl
96 reactions*	2x 432 µl (2x 48x 9 µl)	2x 24 µl	2x 624 µl	2x 1,120 µl

* When pooling over 56 RT reactions, split the pool into two parallel purification reactions.

5 Pool 9 µl of each RT reaction (a minimum of 8 RT reactions) in a fresh PCR plate or 1.5 ml tube and note the final volume of the pool. Fresh pipette tips do not have to be used when pooling RT reactions into the same well / tube. **ATTENTION:** If evaporation occurs and the total volume of the pool is lower than expected, fill up to the expected volume using Elution Buffer (**EB**).

6 Add 24 µl of Purification Beads (**PB**) and the required volume of Purification Solution (**PS**) to the pooled reaction. Mix well, spin down briefly, and incubate for 5 minutes at room temperature.

7 Place the plate (if only 8 RT reactions were pooled) or 1.5 ml tube onto a magnet and let the beads collect for 5 - 10 minutes or until the supernatant is completely clear. **REMARK:** When using 1.5 ml tubes, look vertically into the tubes to ensure collection of **PB** on the magnet.

8 Remove and discard the clear supernatant without removing the PCR plate or 1.5 ml tube from the magnet. Make sure that accumulated beads are not disturbed.

9 Add 80 % EtOH 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.

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 could negatively influence the elution and the resulting library yield.

Add 12 µl of Elution Buffer (**EB**) per well / tube, remove the plate / tube 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 beginning, quickly spin down the plate / tube to make sure all liquid is collected at the bottom.

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

16 Incubate for 10 minutes at 95 °C, then cool down to 25 °C. Spin down and 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 at a reduced ramp speed of 0.5 °C/second. Incubate the reaction for 3 minutes at 25 °C. Quickly spin down the plate / tube before removing the sealing foil.

19

Add 1 μ l of **E2** per reaction. Mix well and spin down. **REMARK:** Use a pipette set to 15 μ l 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 μ l 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 μ l 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 μ l 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 μ l 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 could 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.

4.2 Library Amplification

Preparation

PCR		Purification (Cat. No. 022)
PM ○ - thawed at RT P5* ● - thawed at RT P7* ● - thawed at RT PE ○ - 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 see Appendix B, p.21.	Equilibrate all reagents to room temperature for 30 minutes prior to use.

* Primers P5 and P7 need to be replaced by barcoded primers containing 12 nt UDIs (Cat. No. 101 - 105, 156) 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 (**PM** ○) 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, we recommend Lexogen's 12 nt UDI Sets (Cat. No. 101 - 105, 156) with pre-mixed unique dual indexes. For further information on multiplexing, see Appendix E, p.26.

Between 18 and 22 nucleotides of Read 2 must 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 endpoint PCRs 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.
- **Before proceeding, please review Appendix A, p.18 and Appendix B, p.21.**
- The PCR Add-on Kit for Illumina (Cat. No. 020) is required if you have 7 or more pools.
- The PCR Add-on Kit (Cat. No. 020) **cannot** be used for Endpoint PCR.

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 mastermix 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 pool.

OPTIONAL: Non-indexed PCR primer **P5** and non-indexed PCR primer **P7** may be substituted using 10 µl of pre-mixed i5 / i7 12 nt UDI primers available as Cat. No. 101 - 105, 156 at 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 could 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 D, p.24). See Appendix E, p.26 for multiplexing of pools. 🛑 Safe stopping point. Libraries can be stored at -20 °C at this point.

5. 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.9)!
- ☐ Incubate for 3 min at 85 °C, then cool to 25 °C.
- ☐ Prepare a mastermix with 2.5 µl **FS** ●, 0.25 µl **DTT** ● and 0.25 µl **E1** ● per RT reaction, mix well, aliquot to 8-tube strip. Do not cool the mastermix. Keep it at room temperature.
- ☐ Add 3 µl **FS / DTT / E1** mix per RT reaction, mix well.
- ☐ 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 required volume of **PS** (see p.10), mix well, and incubate 5 min at room temp.
- ☐ Place on magnet for 5 - 10 min, discard supernatant.
- ☐ Rinse beads twice with 80 % EtOH (see p.10), 30 sec.
- ☐ Air dry beads for 5 - 10 min. **ATTENTION:** Do not let the beads dry too long!
- ☐ Add 12 µl **EB**, remove from magnet, mix well, incubate 2 min at room temperature. **OPTIONAL:** Individual purifications can be combined again at this step (see p.11).
- ☐ 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 O**, 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 room temperature.
- ☐ Place on magnet for 2 - 5 min, discard supernatant.
- ☐ Add 40 µl **EB**, remove from magnet, mix well, incubate 2 min at room temperature.
- ☐ Add 52 µl **PS**, mix well, incubate 5 min at room temperature.
- ☐ 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 room temperature.
- ☐ Place on magnet for 2 - 5 min, transfer 17 µl of the supernatant into a fresh PCR plate.
👉 Safe stopping point.

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 pool. **OPTIONAL:** Replace **P5** and/or **P7** 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, 15 sec
 - 60 °C, 15 sec
 - 72 °C, 60 sec
 } **9 - 15x**
 (see Appendix B, p.21)
- 72 °C, 6 min
- 10 °C, ∞. 🛑 Safe stopping point.

Purification

- ☐ Add 31.5 µl **PB** per PCR reaction, mix well, incubate 5 min at room temperature.
- ☐ Place on magnet for 2 - 5 min, discard supernatant.
- ☐ Add 30 µl **EB**, remove from magnet, mix well, incubate 2 min at room temperature.
- ☐ Add 30 µl **PS**, mix well, incubate 5 min at room temperature.
- ☐ 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 room temperature.
- ☐ Place on magnet for 2 - 5 min, transfer 15 - 17 µl of the supernatant into a fresh PCR plate.
- 🛑 Safe stopping point.

6. Appendix A: RNA Input, Pooling 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 with 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 sample. This input amount allows full flexibility for pooling and multiplexing. A minimum of 8 and a maximum of 96 samples can be pooled after reverse transcription.
- The minimum recommended input amount of total RNA is 10 ng per sample.
- The maximum recommended input amount of total RNA is 120 ng per sample. When using 120 ng total RNA input, a maximum of 8 samples can be pooled.
- The input amount of RNA per sample determines the pooling capacity. The maximum amount of cDNA corresponding to 960 ng total RNA input can be processed in one second strand synthesis pooled reaction. Refer to the table on p.10, or our online [QuantSeq-Pool Calculator](#) for details.
- Lower RNA inputs (<10 ng) are not suitable for QuantSeq-Pool.

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 four 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, 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), and SIRV-Set 4 (Cat. No. 141) contains the long SIRV module with 15 RNAs of 4 - 12 kb length in addition to the SIRV isoforms and ERCC transcripts of SIRV-Set 3. 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.

Pooling Guidelines

- When pooling RT reactions samples at step **5**, the volumes of Purification Solution (**PS**) and 80% EtOH need to be adjusted.
- The online [QuantSeq-Pool Calculator](#) tool calculates these volumes automatically, as well as the expected total volume of the pool after entering the number of samples you intend to pool.
- The volumes of **PS** and 80% EtOH to add are calculated using the formulas below.
ATTENTION: The volume of 80% EtOH to add differs if only 8 RT reactions are pooled (3b), versus pooling >8 RT reactions (3a):

1. Volume of Pool (μl) = 9 x number of RT reactions pooled
 2. Volume of **PS** to add (μl) = (Volume of Pool x 1.5) - 24
 - 3a. Volume of 80% EtOH to add (μl) if pooling >8 RT reactions
= Volume of Pool + Volume of **PS** + Volume of **PB** + 40 μl
- OR**
- 3b. Volume of 80% EtOH to add (μl) if pooling 8 RT reactions
= Volume of Pool + Volume of **PS** + Volume of **PB**

PCR Cycles

- **The optimal cycle number for your specific sample type should be determined using the qPCR assay** (see Appendix B, p.21).
- 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).
- It is sufficient to assess each sample type once and refer to the determined optimal cycle numbers also for future experiments.
- If you have ≥ 7 pools, the PCR Add-on Kit (Cat. No. 020.96) is required for qPCR.
- If you have ≤ 6 pools, the provided solutions for Library Amplification (**PM, P5, P7, PE**) are sufficient for qPCR and final amplification.
- The PCR Add-on Kit (Cat. No. 020.96) **cannot** be used for Endpoint PCR (steps 34 – 36).

ATTENTION: Be careful not to overcycle your library pool! If the determined number of cycles for the Endpoint PCR exceeds the maximum for the input amount indicated in the table below, please contact support@lexogen.com for advice before proceeding to library amplification.

Amount of Total RNA Equivalent per Second Strand Synthesis Pool	Number of Cycles for Endpoint PCR for UHRR
80 ng (8x 10 ng)	12 – 15
240 ng (e.g., 12x 20 ng or 24x 10 ng)	11 – 14
480 ng (e.g., 24x 20 ng or 48x 10 ng)	10 – 13
960 ng (e.g., 8x 120 ng, 48x 20 ng, or 96x 10 ng)	9 – 12

7. Appendix B: 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 A, p.18). **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 D, p.24).

qPCR to Determine the Optimal Cycle Number for Endpoint PCR

The qPCR assay is used to determine optimal cycle numbers for subsequent dual or single indexing Endpoint PCR.

When preparing ≤ 6 pools, the provided solutions for Library Amplification (**PM** ○, **P5** ●, **P7** ●, and **PE** ○) are sufficient for both qPCR and final library amplification. Alternatively, the PCR Add-on Kit for Illumina (Cat. No. 020: PCR Mix (**PCR** ●), Enzyme Mix (**E** ●), and the P7 Primer (**7000** ●)) is only required if you have ≥ 7 second strand synthesis pools.

In addition, SYBR Green I nucleic acid dye (Sigma Aldrich, S9430 or ThermoFisher, Cat. No. S7585) is required 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. The inclusion of passive reference dyes such as ROX is neither required nor recommended. For further assistance contact support@lexogen.com.

1 Dilute the double-stranded library from step 24 to 19 μ l by adding 2 μ l 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.

For each reaction combine: 1.7 μ l of cDNA with 7 μ l PCR Mix (**PM** ○), 5 μ l **P5** ●, 5 μ l **P7** ●, and 1 μ l Enzyme Mix (**PE** ○), 1.2 μ l of 2.5x SYBR Green I nucleic acid stain, and 14.1 μ l of **EB**, per reaction. Mix well.

3 **OPTION:** Alternatively, using PCR Add-on Kit reagents: mix 1.7 μ l of cDNA with 7 μ l of PCR Mix (**PCR** ●), 5 μ l of P7 Primer (**7000** ●), 1 μ l of Enzyme Mix (**E** ●), 1.2 μ l of 2.5x SYBR Green I nucleic acid dye and 14.1 μ l 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).

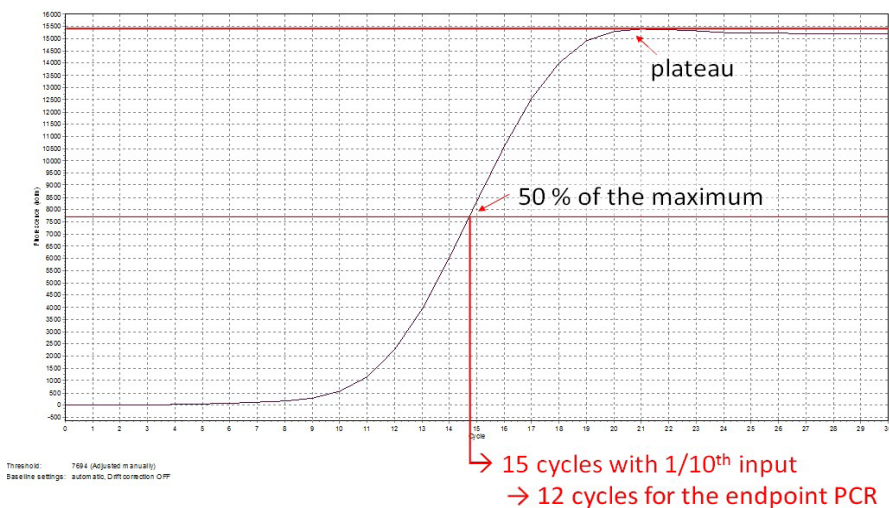


Figure 3. Calculation of the number of cycles for the endpoint PCR.

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.

8. Appendix C: Library Reamplification

Reamplification of QuantSeq-Pool Libraries

If your library yields are extremely low and insufficient for pooling, reamplification can be performed. The Reamplification Add-on Kit for Illumina (080.96) is required for the reamplification of QuantSeq-Pool libraries. This kit is available only upon request.

ATTENTION: The PCR Add-on Kit (Cat. No. 020) **cannot** be used for reamplification of dual-indexed QuantSeq-Pool libraries (i.e., libraries prepared with UDIs). Please contact Lexogen at support@lexogen.com for more information.

9. Appendix D: 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 pools 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 E, 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 library pool 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). Please note to utilize a high sensitivity chip with good resolution to resolve larger fragments up to at least 6,000 basepairs.

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-Pool library pool 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.

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)). When using UHRR the majority of inserts are longer than ~430 bp in size, corresponding to final library fragment sizes ≥ 600 bp, and the yield after amplification is typically 1 - 3 ng/ μ l (see Figure 4). Library shape and size distribution may appear different depending on the microfluidics platforms used for quality control e.g., TapeStation traces may appear shorter than those from a Bioanalyzer. If you are unsure about the size distribution or yield of your amplified libraries, please contact support@lexogen.com before proceeding to sequencing.

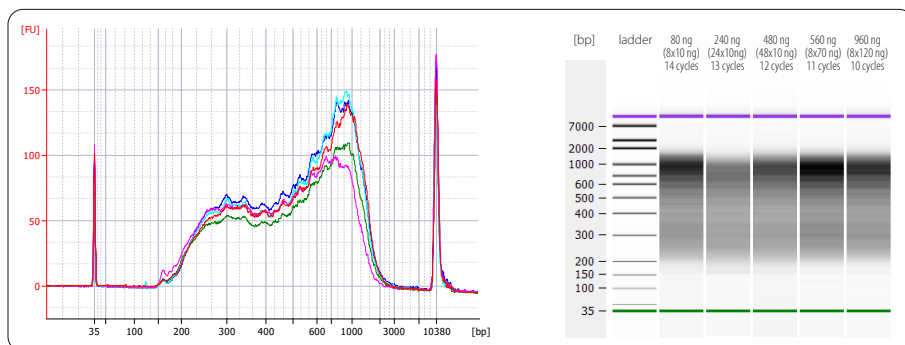


Figure 4. Bioanalyzer traces of QuantSeq-Pool libraries prepared from different input amounts of total RNA (UHRR) and pooling various amounts of total RNA equivalents after reverse transcription. Libraries were prepared following the protocol described in this user guide, pooling either 8 x 10 ng (red trace, 14 PCR cycles, 2.52 ng/ μ l yield), 24 x 10 ng (pink trace, 13 PCR cycles, 2.18 ng/ μ l yield), 48 x 10 ng (green trace, 12 PCR cycles, 2.25 ng/ μ l yield), 8 x 70 ng (cyan trace, 11 PCR cycles, 3.17 ng/ μ l yield), or 8 x 120 ng (blue trace, 10 PCR cycles, 2.51 ng/ μ l yield) at step 5. RNA removal, second strand synthesis, and library pool amplification were 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. The library yield quantification corresponds to a range of 200 - 2,000 bp in the Bioanalyzer traces.

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 B, p.21. 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.

If you suspect your libraries to be overcycled but are not sure, please contact support@lexogen.com before proceeding to sequencing.

10. Appendix E: 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 indexes (UDIs) for the individual pools (containing up to 96 sample-barcode 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's 12 nt Unique Dual Indexing Sets for up to 384 UDIs (Cat. No. 101 - 105, 156) with pre-mixed i5 and i7 indices in a convenient 96-well format are available and offer superior error correction capacity for massive multiplexing. For more information on indexing, please visit our website and our Frequently Asked Questions (FAQs) or contact support@lexogen.com.

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.).

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 180 - 9,000 bp (LL peak at ~169 bp). For 6 nt single indexing (i7) select 180 - 9,000 bp (LL peak at ~175 bp), and for 12 nt unique dual indexing use 200 - 9,000 bp (LL peak at ~197 bp). An elevated base line that extends past the upper marker is an indication of overcycling, and quantification will be biased.

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

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


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 between 169 - 197 bp, depending on the indexing method utilized. Linker-linker artifacts should not compose more than 0 - 3 % of the total lane mix for sequencing. If the fraction of linker-linker or other small fragments 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 (H₂O).
 - 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.15).

11. Appendix F: 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 i1 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 poly(T) 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). The i7 Index Primer is always used for Index Read 1 (i7) sequencing. Index Read 2 sequencing is exemplified for the i5 Index 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
...Insert)-i1(12 nt)-N(i1) AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGCTTCTGCTTG 3'
...Insert)-i1(12 nt)-N(i1) TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
3'-(Read 2 Sequencing Primer)-5'
```

Read 1 for QuantSeq-Pool libraries:

i7 Index Primer (not supplied):

5'ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

Read 2 for QuantSeq-Pool libraries (required for UMI and i1 sample-barcode read out):

i5 Index Primer (not supplied):

5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

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.

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

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

Index 2 Read (i5): i5 Index 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).

12. Appendix G: 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. A free script for QuantSeq-Pool data analysis is also available on the Lexogen GitHub page (https://github.com/Lexogen-Tools/quantseqpool_analysis). For additional information, please refer to www.lexogen.com/quantseq-data-analysis or check the online Frequently Asked Questions (FAQs).

Read Orientation

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.

Demultiplexing i5 / i7

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

In addition 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.32). 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-barcode libraries can be performed using Lexogen's Demultiplexing Tool (available free of charge at <https://github.com/Lexogen-Tools>). 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 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. 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 nucleotide long 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 de-duplication, e.g., `umi_tools`.

13. Appendix H: Revision History

Publication No. / Revision Date	Change	Page
139UG272V0300 Nov. 22, 2021	Changes to kit composition. Addition of DTT.	6
	Protocol update, Step 2: Cool to 25 °C after denaturation instead of 42 °C. No pre-warming of mastermix required.	9, 16
	Protocol update, Step 3: Prepare a mastermix of 2.5 µl FS + 0.25 µl DTT + 0.25 µl E1 (formerly: 2.75 µl FS + 0.25 µl E1) per reaction.	9, 16
	Attention notes and Appendix information regarding qPCR and Endpoint PCR updated.	14, 20
	Updated data analysis recommendations.	31
139UG272V0200 Feb. 18, 2021	Updated terminology for describing individual samples and pooled reactions.	all
	Removed General Guidelines.	8
	Included QuantSeq-Pool calculator link for determining purification reagent volumes.	8, 10, 19
	Updated text and phrasing in steps 3 to 5.	9, 10
	Incubation time on magnet changed from 2 - 5 minutes to 5 - 10 minutes in step 7.	10
	Elution volume changed to 12 µL in step 12.	11
	Streamlined information in Appendix sections.	18 - end
	Added pooling guidelines in Appendix A.	19
	Corrected linker-linker sizes in Appendix E.	27 - 28
139UG272V0100 Aug. 10, 2020	Initial Release.	

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)
080 (Reamplification Add-on Kit for Illumina)
101 - 104 (Lexogen UDI 12 nt Set A1 - A4)
105 (Lexogen UDI 12 nt Set B1)
156 (Lexogen UDI 12 nt A1 - A4)

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

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