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QUANT™ SEQ FFPE
Sequencing that counts

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

User Guide

Catalog Numbers:
222 (QuantSeq FFPE 3' mRNA-Seq Library Prep Kit FWD with UDI 12 nt Set A1, or A1-A4)
223 (QuantSeq FFPE 3' mRNA-Seq Library Prep Kit FWD with UDI 12 nt Set B1)

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1. Overview

Lexogen's QuantSeq FFPE 3' mRNA-Seq Library Prep Kit Forward (FWD) with 12 nt Unique Dual Indices (UDIs) enables library preparation to generate Illumina-compatible libraries from polyadenylated RNA within 4.5 hours. The QuantSeq protocol generates only one fragment per transcript, resulting in extremely accurate gene expression values, and the sequences obtained are close to the 3' end of transcripts.

QuantSeq uses total RNA as input, hence no prior poly(A) enrichment or rRNA depletion is required. Information on input requirements can be found in Appendix A, p.16. Library generation is initiated by oligo(dT) priming (Fig. 1). The primer already contains a partial Illumina-compatible linker sequence plus a 12 nt Unique Molecular Index (UMI). After first strand synthesis, the RNA is removed and second strand synthesis is initiated by random priming. The random primer also contains a partial Illumina-compatible linker sequence. No purification is required between first and second strand synthesis. The insert size is optimized for shorter read lengths: SR50 - 100, with a partial Read 2 readout to access the UMI information.

Second strand synthesis is followed by a magnetic bead-based purification step. The library is then amplified, introducing the sequences required for cluster generation. Lexogen's 12 nt Unique Dual Indices for multiplexing are included in the kit and are introduced during the PCR amplification step (Set A: Cat. No. 222, or Set B: Cat. No. 223). Lexogen UDI 12 nt Sets are also available as separate Unique Dual Indexing V2 Add-on Kits (Cat. No. 198 - 203) for use with other library preps.

QuantSeq FFPE FWD contains the Read 1 linker sequence in the second strand synthesis primer, hence NGS reads are generated towards the poly(A) tail and directly correspond to the mRNA sequence. To pinpoint the exact 3' end, longer reads may be required. For UMI read out (optional) an assymetric paired-end sequencing is required with a Read 2 of 12 nt. Sequencing longer in Read 2 (e.g., to determine the 3' end) is feasible, however reading through the poly(T) stretch and sequencing through the homopolymer stretch, reduces the overall quality of Read 2.

QuantSeq FWD 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. The kits include magnetic beads for the purification steps and hence are compatible with automation.

Additional information regarding quality control, add-on modules, multiplexing, and sequencing guidelines are found in Appendix A, p.16 - Appendix D, p.22

Each purchased QuantSeq kit provides a code for free data analysis, including differential expression (DE) analysis on Lexogen's web-based data analysis platform Kangooroo (for more details, please read Appendix E, p.24).

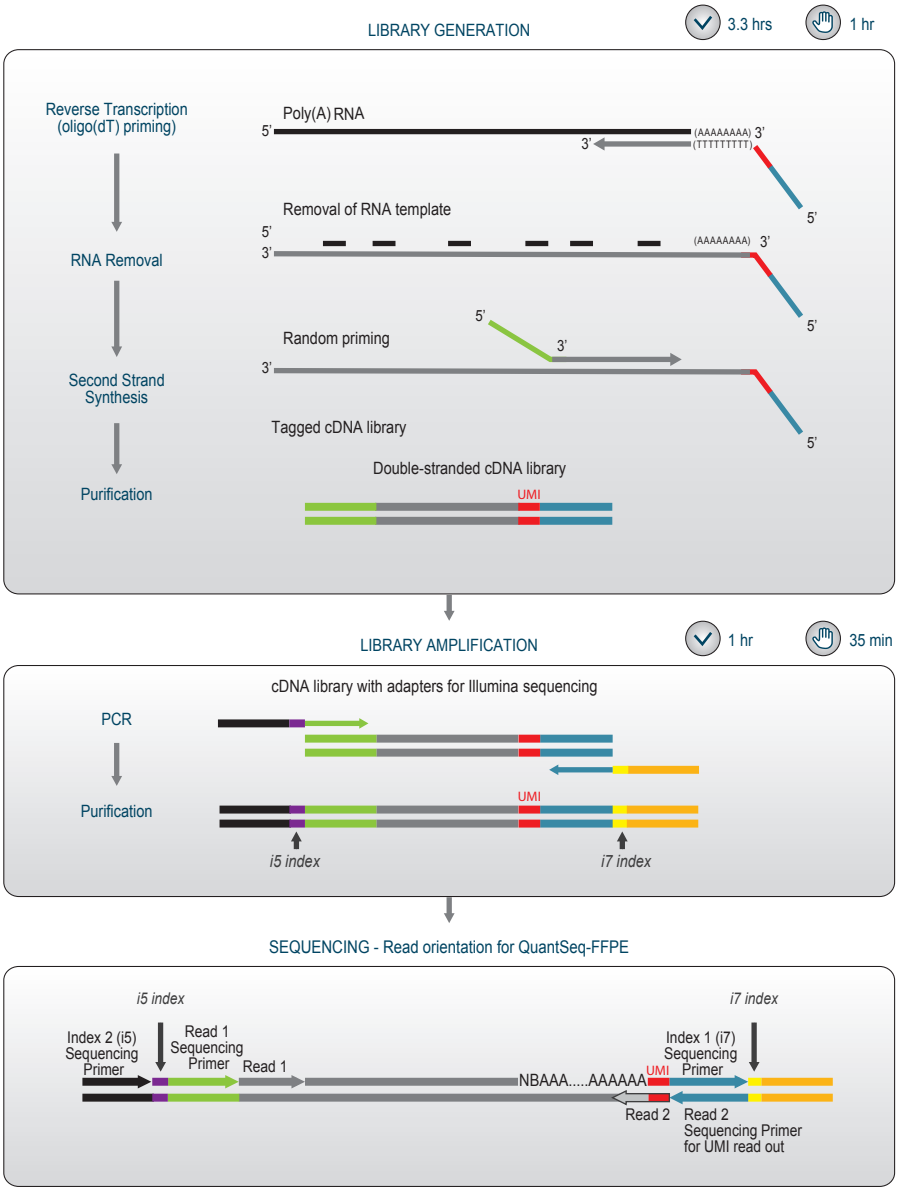

Figure 1. Schematic overview of the QuantSeq FFPE 3′ mRNA-Seq FWD with UDIs library preparation workflow. Sequencing read orientation for QuantSeq FFPE FWD is depicted, where Read 1 reflects the mRNA sequence. Limited Paired-end sequencing (R2: 12 nt) is required for UMI read out (beginning of Read 2).

2. Kit Components and Storage Conditions

Upon receiving the QuantSeq kit, store the Purification Module (Cat. No. 022) containing **PB**, **PS**, and **EB** at +4 °C, and the rest of the kit in a -20 °C freezer. **NOTE:** Before use, check the contents of **PS**. If a precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

Kit Component	Tube / Plate Label	Volume*			Storage
		24 preps	96 preps	384 preps	
Molecular Biology Grade Water	**H_2O** ●	79.2 µl	316.8 µl	4x 316.8 µl	-20 °C
UMI Oligo(dT) primer FWD	**UMI dT** ●	26.4 µl	105.6 µl	4x 105.6 µl	-20 °C
10x First Strand cDNA Synthesis Mix	**10xFS** ●	26.4 µl	105.6 µl	4x 105.6 µl	-20 °C
DTT	**DTT** ●	6.6 µl	26.4 µl	4x 26.4 µl	-20 °C
Enzyme Mix 1	**E1** ●	6.6 µl	26.4 µl	4x 26.4 µl	-20 °C
RNA Removal Solution	**RS** ○	66 µl	264 µl	4x 264 µl	-20 °C
Removal Enzyme	**RE** ○	13.2 µl	52.8 µl	4x 52.8 µl	-20 °C
Second Strand Synthesis Mix 1	**SS1** ●	132 µl	528 µl	4x 528 µl	-20 °C
Second Strand Synthesis Mix 2	**SS2** ●	39.6 µl	158.4 µl	4x 158.4 µl	-20 °C
Enzyme Mix 2	**E2** ●	13.2 µl	52.8 µl	4x 52.8 µl	-20 °C
Library Amplification Module					
PCR Mix	**PM** ○	184.8 µl	739.2 µl	2x 1,478.4 µl	-20 °C
PCR Enzyme Mix	**PE** ○	26.4 µl	105.6 µl	422.4 µl	-20 °C
Lexogen UDI 12 nt Sets					
Lexogen UDI 12 nt Set A1 or B1	**UDI12A_0001-0024, UDI12B_0001-0024**	10 µl / rxn			-20 °C
Lexogen UDI 12 nt Set A1 or B1	**UDI12A_0001-0096, UDI12B_0001-0096**		10 µl / rxn		-20 °C
Lexogen UDI 12 nt Sets A1-A4	**UDI12A_0001-0384**			10 µl / rxn	-20 °C
Purification Module					
Purification Beads	**PB**	1,320 µl	5,280 µl	4x 5,280 µl	+4 °C
Purification Solution	**PS**	2,693 µl	10,772 µl	4x 10,772 µl	+4 °C
Elution Buffer	**EB**	2,904 µl	11,616 µl	4x 11,616 µl	+4 °C

*including ≥10 % surplus

3. User-Supplied Consumables and Equipment

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.

ATTENTION: Before starting this protocol, please read the General Guidelines for Lexogen Kits, which are available online. These provide a detailed overview of RNA and kit component handling, as well as general RNA input requirements.

Reagents / Solutions

- 80 % fresh ethanol (**EtOH**, for washing of Purification Beads, **PB**).
- Lexogen PCR Add-on and Reamplification Kit V2 for Illumina (Cat. No. 208), for qPCR assay.
- Recommended: SYBR Green I (Sigma-Aldrich, Cat. No. S9430 or ThermoFisher, Cat. No. S7585) 10,000x 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 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 B, p.18 for information on library quantification methods.

4. Detailed Protocol

4.1. Library Generation

Preparation

First Strand cDNA Synthesis	RNA Removal	Second Strand Synthesis	Purification
H_2O ● – stored at -20 °C **10xFS** ● – thawed at RT **UMI dT** ● – thawed at RT **DTT** ● – thawed at RT **E1** ● – keep on ice or at -20 °C	**RS** ○ – thawed at RT **RE** ○ – keep on ice or at -20 °C	**SS1** ● – thawed at 37 °C **SS2** ● – thawed at RT **E2** ● – keep on ice or at -20 °C	**PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – stored at +4 °C
95 °C, 1 min place on ice; 42 °C, 15 min	37 °C, 15 min 95 °C, 10 min cool to 25 °C	98 °C, 1 min, then cool to 25 °C (0.2 °C/sec) 25 °C, 15 min; 25 °C, 15 min	Equilibrate all reagents to room temperature for 30 min prior to use.

First Strand cDNA Synthesis - Reverse Transcription

An oligo(dT) primer containing an Illumina-compatible sequence at its 5' end and a 12 nt Unique Molecular Index (UMI) between oligo(dT) and Illumina linker is hybridized to the RNA and reverse transcription is performed.

ATTENTION: The minimum recommended input amount for QuantSeq FFPE is 10 ng. Input amounts ≤10 ng may also be used, but results depend on the quality of the FFPE RNA.

1. Mix 10 ng - 200 ng of total FFPE RNA in a volume of up to 4.5 µl, with 1 µl UMI oligo(dT) FWD primer (**UMI dT** ●). If necessary (e.g., less than 5 µl input RNA was added), adjust the total volume to 5.5 µl with Molecular Biology Grade Water (**H_2O** ●). Mix well by pipetting. Ensure the plate is tightly sealed, and spin down to collect the liquid at the bottom of the wells. **OPTIONAL:** Total RNA input volume can be increased to up to 7.5 µl for manual preps, if omitting the 3 µl **H_2O** ● from the **10xFS** / **DTT** / **E1** mastermix in step 3.
2. Denature the RNA / **UMI dT** mix for 1 minutes at 95 °C in a thermocycler and then place on ice.
3. Prepare prepare a mastermix containing 3 µl **H_2O** ●, 1 µl **10xFS** ●, 0.25 µl **DTT** ●, and 0.25 µl **E1** ● per sample. Mix well, spin down, and pre-warm the mastermix for 2 - 3 minutes at 42 °C. **OPTIONAL:** If 7.5 µl RNA were used, omit adding 3 µl **H_2O** ● to the mastermix.

4 Spin down the denatured RNA / **UMI dT** samples from step 2 at room temperature to make sure all liquid is collected at the bottom of the wells. Place the samples onto the thermocycler at 42 °C and carefully remove the sealing foil. Add 4.5 µl of the **H_2O / 10xFS** / **DTT** / **E1** mastermix or 1.5 µl of the **10xFS** / **DTT** / **E1** (if 7.5 µl RNA input was used), to each reaction mix well, and seal the plate. Spin down briefly and incubate the reactions for 15 minutes at 42 °C. **REMARK:** For automation we recommend pipetting 4.5 µl of the mastermix, hence using a maximum RNA input volume of 4.5 µl. **ATTENTION:** Briefly spin down the samples and proceed immediately to step 5. Do not cool the samples below room temperature after reverse transcription.

RNA Removal

During this step the RNA template is degraded. This is essential for efficient second strand synthesis. Before removing the sealing foil after the first strand synthesis reaction, quickly spin down the plate to make sure all liquid is collected at the bottom of the wells.

5 Prepare a mastermix of 2.5 µl RNA Removal Solution (**RS** ○) and 0.5 µl Removal Enzyme (**RE** ○) per reaction. Mix well and spin down.

6 Add 3 µl of the **RS /RE** mastermix directly to the first strand cDNA synthesis reaction. Mix well and reseal the plate using a fresh foil and spin down.

7 Incubate for 15 minutes at 37 °C, followed by 10 minutes at 95 °C, then cool down to 25 °C. Spin down and carefully remove the sealing foil. Proceed immediately to step 8.

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: **SS1** ● 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 13 at room temperature to give it at least 30 minutes to equilibrate.

8 Add 5 µl Second Strand Synthesis Mix 1 (**SS1** ●) to the reaction. Mix well by pipetting, seal the plate, and spin down. **REMARK:** Use a pipette set to 15 µl for efficient mixing.

9 Incubate for 1 minute at 98 °C in a thermocycler, and slowly cool down to 25 °C at a reduced ramp speed of 0.2 °C/second. Incubate the reaction for 15 minutes at 25 °C. Quickly spin down the plate before removing the sealing foil.

10 Prepare a mastermix containing 1.5 µl Second Strand Synthesis Mix 2 (**SS2** ●) and 0.5 µl Enzyme Mix 2 (**E2** ●). Mix well. **ATTENTION:** Keep the mastermix at room temperature.

11 Add 2 µl of the **SS2** / **E2** mastermix per reaction. Mix well and spin down.

12 Incubate for 15 minutes at 25 °C, then briefly spin down. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The double-stranded library is purified using magnetic beads to remove all reaction components. The Purification Module (**PB**, **PS**, and **EB**) should equilibrate for 30 minutes at room temperature before use. The Purification Beads (**PB**) must be fully resuspended before use. Thorough mixing by pipetting or vortexing is recommended.

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

13 Add 8 µl of Purification Beads (**PB**) to each reaction. Mix well and incubate for 5 minutes at room temperature.

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

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

16 Remove the plate from the magnet, add 20 µl of Elution Buffer (**EB**), and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.

17 Add 26 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature.

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

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

20 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.

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

22 Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Do not let the beads dry too long!

23 Remove the plate from the magnet, add 20 µl of Elution Buffer (**EB**), and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.

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

25 Transfer 17 µl of the clear supernatant into a fresh PCR plate. Do not transfer any beads. **ATTENTION:** If a qPCR assay is performed to determine the exact number of cycles required for the endpoint PCR (using Cat. No. 208), add an additional 2 µl of Elution Buffer (**EB**) to the eluted library. For further details please refer to 208UG591. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

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

This section describes unique dual indexing PCR for multiplexing up to 384 libraries using the Lexogen 12 nt UDIs included in the QuantSeq FFPE 3′ mRNA-Seq kits.

Preparation

PCR				Purification (Cat. No. 022)*	
PM ○ **PE** ○ **Lexogen UDI 12 nt Sets** (A1 - A4, or B1)		- thawed at RT - keep on ice or at -20 °C - thawed at RT	**spin down before opening!**	**PB** **PS** **80 % EtOH** **EB**	– stored at +4 °C – stored at +4 °C – provided by user **prepare fresh!** – 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, ∞	12- 26x Endpoint cycle number as determined by qPCR (Cat. No. 208).		Equilibrate all reagents to room temperature for 30 min prior to use.	

PCR

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

ATTENTION: Important notes for Library Amplification.

- **Perform a qPCR assay to determine the optimal PCR cycle number for endpoint PCR**. The number of PCR cycles for library amplification must be adjusted according to RNA input amount, quality, and sample type. The PCR Add-on and Reamplification Kit V2 for Illumina (Cat. No. 208) is required.
- Avoid cross contamination when using the Lexogen UDI 12 nt Sets. Spin down the Index Set before opening and visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired UDIs only. Reseal opened wells with a fresh sealing foil after use to prevent cross contamination.
- Each well of the Lexogen UDI 12 nt Set is intended for single use only.

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

26	Prepare a mastermix containing 7 µl PCR Mix (**PM** ○) and 1 µl PCR Enzyme Mix (**PE** ○) per reaction.
27	Add 8 µl of the **PM / PE** mastermix to 17 µl of the eluted library.
28	Add 10 µl of the respective Unique Dual Index Primer pair (UDI12A_0001-0384 or UDI12B_0001-0096) to each sample. Use only one UDI per sample! **ATTENTION:** Spin down the plates containing the UDIs before opening! Pierce or cut open the sealing foil of the wells containing only the desired UDIs. Reseal opened wells of the UDI plate after use with a fresh sealing foil to prevent cross contamination!
29	Conduct 12 - 26 cycles of PCR (as determined by qPCR using Cat. No. 208) with the following program: Initial denaturation for 60 seconds at 95 °C, then 12 - 26 cycles of 15 seconds at 95 °C, 15 seconds at 60 °C and 60 seconds at 72 °C, and a final extension for 6 minutes at 72 °C, 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.

30	Add 31.5 µl of Purification Beads (**PB**) to each reaction. Mix well and incubate for 5 minutes at room temperature.
31	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
32	Remove and discard the clear supernatant without removing the PCR plate from the magnet. Do not disturb the beads!
33	Remove the plate from the magnet, add 20 µl of Elution Buffer (**EB**), and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
34	Add 20 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature.

35	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
36	Remove and discard the clear supernatant without removing the plate from the magnet. Do not disturb the beads.
37	Add 120 µl of 80 % EtOH, and incubate the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.
38	Repeat this washing step once for a total of two washes. **ATTENTION:** Remove the supernatant completely.
39	Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Do not let the beads dry too long!
40	Remove the plate from the magnet, add 20 µl of Elution Buffer (**EB**), and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
41	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
42	Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Do not transfer any beads. Libraries are now finished and ready for quality control (Appendix B, p.18), pooling (for multiplexing, Appendix C, p.20), and cluster generation. ☞ 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
☐	Mix 4.5 µl RNA and 1 µl **UMI dT** ●. **OPTIONAL:** Up to 7.5 µl RNA can be added, if omitting water or this from the subsequent mastermixing.
☐	Incubate for 1 min at 95 °C, then place on ice.
☐	Prepare a mastermix with 3 µl **H_2O** ●, 1 µl **10xFS** ●, 0.25 µl **DTT** ●, and 0.25 µl **E1** ● per reaction, mix well and pre-warm for 2 - 3 min at 42 °C. Place samples at 42 °C. **OPTIONAL:** If 7.5 µl RNA are used do not add **H_2O** ● to the mastermix.
☐	Add 4.5 µl of the **H_2O / 10xFS / DTT/ E1** mastermix per reaction, mix well, and spin down. **Keep samples on thermocycler at 42 °C when adding mastermix!** **NOTE:** If **H_2O** ● was omitted from the mastermix because of higher RNA input volume add only 1.5 µl of an **10xFS / DTT/ E1 mastermix** at this step.
☐	Incubate for 15 min at 42 °C. **Proceed immediately to RNA Removal!**
	RNA Removal
☐	Prepare a mastermix of 2.5 µl **RS** ○ and 0.5 µl **RE** ○, mix well.
☐	Add 3 µl **RS / RE** per reaction, mix well.
☐	Incubate 15 min at 37 °C, then 10 min at 95 °C, cool to 25 °C.
	Second Strand Synthesis
☐	Add 5 µl **SS1** ●, mix well.
☐	Incubate 1 min at 98 °C, slowly ramp down to 25 °C (0.2 °C/sec).
☐	Incubate 15 min at 25 °C.
☐	Prepare a mastermix with 1.5 µl **SS2** ● and 0.5 µl **E2** ● per reaction, mix well.
☐	Add 2 µl **SS2 / E2** mix per reaction, mix well.
☐	Incubate 15 min at 25 °C. ☞ Safe stopping point.
	Purification
☐	Add 8 µl **PB** per reaction, mix well, incubate 5 min at RT.
☐	Place on magnet for 2 - 5 min, discard supernatant.
☐	Remove from magnet, add 20 µl **EB**, mix well, incubate 2 min at RT.
☐	Add 26 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!
☐	Remove from magnet, add 20 µl **EB**, mix well, incubate 2 min at RT.
☐	Place on magnet for 2 - 5 min, transfer 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.

ATTENTION: Spin down before opening tubes or plates!

1.2 hrs (+qPCR) Library Amplification

qPCR
[Strongly Recommended! Requires PCR Add-on and Reamplification Kit V2 (Cat. No. 208)]

- ☐ Add 2 µl of **EB** to the 17 µl of eluted cDNA.

  Prepare a 2.5x stock of SYBR Green I nucleic acid stain (i.e., 1:4,000 dilution in DMSO; use Sigma-Aldrich, Cat. No. S9430 or ThermoFisher, Cat. No. S7585).
- ☐ Combine 1.7 µl of cDNA with: 7 µl **PM** ○, 5 µl **P5** ●, 5 µl **P7** ●, 1 µl **PE** ○, 1.4 µl of 2.5x SYBR Green I nucleic acid stain, and 13.9 µl of **EB**, per reaction. Mix well.
- ☐ PCR: 95 °C, 60 sec

  95 °C, 15 sec
  60 °C, 15 sec } **35x**
  72 °C, 60 sec

  72 °C, 6 min

  10 °C, ∞. Calculate the optimal cycle number for Endpoint PCR (please refer to 208UG591).

Endpoint PCR

- ☐ Prepare a mastermix with 7 µl PCR Mix (**PM** ○) and 1 µl PCR Enzyme Mix (**PE** ○) per reaction.
- ☐ Add 8 µl of the **PM / PE** mastermix to 17 µl of the eluted library.
- ☐ Add 10 µl of one Unique Dual Index Primer pair (UDI12A_0001-0384, or UDI12B_0001-0096) to each sample. **ATTENTION:** Reseal opened index wells after use! Use only one UDI / sample.
- ☐ PCR: 95 °C, 60 sec

  95 °C, 15 sec
  60 °C, 15 sec } **12 - 26x**
  72 °C, 60 sec

  72 °C, 6 min

  10 °C, ∞. ☞ Safe stopping point.

Purification

- ☐ Add 31.5 µl **PB** per reaction, mix well, incubate 5 min at RT.
- ☐ Place on magnet for 2 - 5 min, discard supernatant.
- ☐ Remove from magnet, add 20 µl **EB**, mix well, incubate 2 min at RT.
- ☐ Add 20 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 min. **ATTENTION:** Do not let the beads dry too long!
- ☐ Remove from magnet, add 20 µl **EB**, mix well, incubate 2 min at RT.
- ☐ Place on magnet for 2 - 5 min, transfer 15 - 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.

6. Appendix A: RNA Input and PCR Cycles

Total RNA isolated from formalin-fixed, paraffin-embedded (FFPE) samples is the intended input for QuantSeq FFPE. No prior rRNA depletion or poly(A) enrichment is required. While also high-quality or fresh frozen RNA can be used as input for QuantSeq FFPE 3′ mRNA-Seq, the protocol is specifically optimized for FFPE inputs protocol. Technically, any total RNA sample that contains polyadenylated mRNA can be used, including e.g., bacterial RNA samples that have been previously polyadenylated.

Quality of FFPE RNA is often assessed by the DV_{200} value. The DV_{200} is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV_{200}, the more degraded the RNA is.
QuantSeq FFPE has been tested extensively with various FFPE input qualities, ranging from DV_{200} 9 % to DV_{200} 50 % and input amounts (5 ng to 200 ng) from multiple tissues (breast, lung, kidney, liver, brain) from mouse as well as human.
While the DV_{200} value can give insights about the fragmentation of the FFPE RNA, other factors such as the preparation of the FFPE slides, the RNA extraction methods, also influence the performance of FFPE RNA in an NGS sample preparation.

Input Guidelines

- The recommended input range for QuantSeq FFPE is 10 ng -200 ng total RNA from FFPE.
- We recommend performing the protocol initially with at least 10 ng total FFPE RNA. RNA inputs ≥50 ng are recommended to detect low abundant transcripts efficiently.
- Lower RNA inputs (≤10 ng) are possible and may lead to higher linker-linker artifacts, especially when using FFPE samples of very low quality.
- **The optimal cycle number for your specific sample type should be determined using the qPCR assay** (using Lexogen's PCR Add-on and Reamplification Kit V2 for Illumina, Cat. No. 208) as the quality of FFPE samples is extremely variable (tissue-dependent differences during fixation, crosslinking, fragmentation).
- The number of PCR cycles optimal for a given input amount of total RNA can vary by up to eight and should be determined for different sample types using the qPCR assay. The table below is provided as a reference only! Optimal cycle numbers could exceed these ranges depending on the sample type (e.g., species, tissue, FFPE RNA quality (DV_{200} value), crosslinks within the RNA, inhibitors in RNA extraction).

Total RNA Input Amount	No. Cycles for Endpoint PCR*
5 ng	17 - 24
10 ng	16 - 22
50 ng	14 - 21
200 ng	12 - 19

* These values are provided as a **reference only!** Sample type influences the optimal cycle number, which should be determined by a qPCR assay (please refer to 208UG591).

ATTENTION:

- FFPE RNA samples are highly variable. Samples with lower mRNA content or lower DV_{200} values may require more PCR cycles. **We strongly recommend performing a qPCR assay** using Lexogen's PCR Add-on and Reamplification Kit V2 for Illumina (Cat. No. 208) to determine the optimal cycle number for library amplification.
- FFPE RNA is highly degraded, hence the insert sizes are smaller than for non-degraded RNA samples (see also Appendix B, p.18). Keep this in mind when choosing your sequencing length.
- If you see that your FFPE RNA generates ~175 bp linker-linker products despite the above-mentioned protocol changes, re-purification of the lane mix with 0.9x **PB** (e.g., 50 µl lane mix plus 45 µl of **PB**, incubating 5 minutes at room temperature, and following the protocol from step 31 on again) may be necessary.
- FFPE RNA can be contaminated with fragmented DNA, which may result in an overestimation of inserted RNA and/or in a high number of intronic and intergenic reads in NGS samples. For FFPE RNA, it is recommended to perform a DNase I treatment, or to distinguish between RNA and DNA when quantifying your input material. Heat inactivation of DNase I should be avoided, and the enzyme should be deactivated by other means such as phenol / chloroform extraction or silica column purification. For best practise, remove DNA prior to library preparation, e.g., with Lexogen's DNA Removal Add-on (Cat. No. 235), or use Lexogen's SPLIT One-Step FFPE RNA Extraction Kit (Cat. No. 236) for RNA Extraction from FFPE material.
- Mitochondrial ribosomal RNAs (mt-rRNAs) are polyadenylated and hence will also be reverse transcribed and converted into a cDNA library if oligo(dT) priming is used during First Strand Synthesis. mt-rRNAs can therefore be observed, but will only represent a minor fraction of the reads when using a 3' mRNA Seq protocol.
- Optionally, an rRNA depletion method, which also removes mt-rRNAs, such as Lexogen's RiboCop rRNA Depletion Kit (Cat. No. 144) can be used before starting the QuantSeq library preparation.
- If adding spike-in controls (e.g., SIRV-Set 3, Cat. No. 051), these should be added prior to any DNase I treatment. For FFPE RNA SIRVs should be spiked in at 0.003 % of the total RNA (0.1 % of presumed 3 % mRNA). For further questions, please contact support@lexogen.com.

7. Appendix B: Library Quality Control

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

Quality Control Methods

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

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

If microcapillary electrophoresis platforms and qPCR machines are not available, very basic quality control can also be performed by separating a small aliquot of the library on a polyacrylamide or agarose gel. Library quantification can also be performed with an inexpensive benchtop fluorometer using one of several commercially available assays, e.g., Qubit dsDNA HS assay. Most UV-Vis spectrophotometers (e.g., NanoDrop, Thermo Fisher Scientific Inc.), are not sensitive enough to accurately quantify NGS libraries at these concentrations and should be avoided.

Typical Results

QuantSeq libraries are intended for a high degree of multiplexing, and hence libraries do not need to be extensively amplified. Library yield, shape, and average insert size may vary, depending on the type of input sample e.g., heavily degraded FFPE samples typically produce shorter libraries than FFPE samples of higher quality (see Figures 4 and 5). The majority of inserts are greater than 75 bp in size, corresponding to final library fragment sizes ≥200 bp.

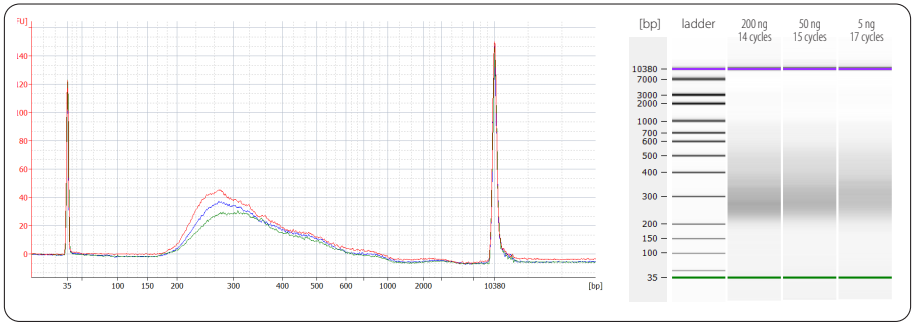

Figure 4. Bioanalyzer traces of QuantSeq FFPE libraries prepared from different input amounts of mouse liver FFPE RNA input. Libraries were prepared using 200 ng (red trace, 14 PCR cycles, 5 nM yield), 50 ng (blue trace, 15 PCR cycles, 4.9 nM yield) and 5 ng (green trace, 17 PCR cycles, 3.3 nM yield). Mm liver FFPE RNA had a DV_{200} of 72 % (RIN 1.2). Endpoint PCR was performed using unique dual indices.

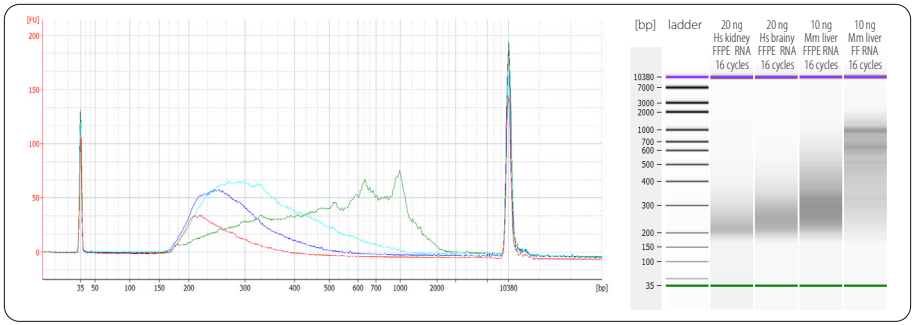

Figure 5. Bioanalyzer traces of QuantSeq FFPE libraries synthesized from 20 ng Hs kidney FFPE RNA (RIN 2.9, DV_{200} 50 %, red trace), 20 ng Hs brain FFPE RNA (RIN 5.5, DV_{200} 91 %, blue trace), 10 ng Mm Liver FFPE RNA (RIN 2.3, DV_{200} 83 %, turquoise trace) and 10 ng Mm Liver fresh frozen RNA (RIN 9.2, green trace). Due to the highly fragmented nature of FFPE RNA libraries are significantly shorter than the ones generated from fresh frozen RNA.

Overcycling

A second peak in high molecular weight regions (between 1,000 - 9,000 bp) is an indication of overcycling. This could occur if cycle numbers are increased too much to compensate for lower input material. Prevent overcycling by using the qPCR assay as described in the PCR Add-on and Reamplification Kit V2 for Illumina User Guide (208UG591).

8. Appendix C: Multiplexing

Libraries prepared with the Lexogen UDI 12 nt Unique Dual Indexing Sets are suitable for sequencing on the Illumina instruments listed below. The Lexogen UDI 12 nt Sets enable adjustable read out of 8, 10, or the full 12 nucleotides long UDI sequence while maintaining superior error correction features. The longer the UDI read-out, the higher the error correction capability. The complete lists of i5 and i7 Index sequences for all Lexogen UDI 12 nt Sets are available at www.lexogen.com/docs/indexing.

Depending on the instrument workflow, flow cell type (paired-end, PE; single-read, SR), and chemistry, i5 Indices are sequenced on the forward or the reverse complement strand.

Illumina Instruments	Flow Cell Type	Workflow	Lexogen UDI 12 nt Unique Dual Indexing
HiSeq 2000 / 2500 HiSeq 3000 / 4000	SR	Forward Strand (A)	**Sets A1 and A1- A4** (UDI12A_0001-0384) QuantSeq FFPE 3′ mRNA-Seq: Cat. No. 220
HiSeq 2000 / 2500 MiSeq NovaSeq 6000 (v1.0 reagent kits)	PE		
iSeq 100 MiniSeq NextSeq 500 - 2000 HiSeq 3000 / 4000 NovaSeq 6000 (v1.5 reagent kits)	PE	Reverse Complement (B)	**Set B1** (UDI12B_0001-0096) QuantSeq FFPE 3′ mRNA-Seq: Cat. No. 223

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

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

Set B1 (UDI12B_0001-0096) for Reverse Complement Workflow (B)

For instruments using the Reverse Complement workflow, the Index 2 Read (i5) is performed after Read 2 Resynthesis, using the Index 2 (i5) Sequencing Primer.

ATTENTION: If QuantSeq FWD preps with UDI Set A (UDI12A_0001-0384) are sequenced on Illumina machines using the Reverse Complement Workflow (B), or *vice versa*, the i5 Index will be read out as reverse complement. **In this case, all 12 nt of the i5 Index must be read out if error correction shall be applied**. Additionally, the reverse complement of the i5 Index read out needs to be analyzed.

EXAMPLE: i512_0001 is read as GTCTTTGGCCCT instead of AGGGCCAAAGAC. The read out in reverse complement (GTCTTTGGCCCT) shall be used for demultiplexing and error correction.

Lane Mix Preparation

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

1. Measure the concentration of each library, using either qPCR or fluorescence-based assays (e.g., Qubit, Thermo Fisher Scientific Inc.).
2. Determine the average library size, using microcapillary electrophoresis analysis (e.g., Bioanalyzer, Agilent Technologies, Inc.). Set the range to 180 - 2,000 bp to include the whole library size distribution, and to exclude any linker-linker artifacts (175 bp), or overcycling bumps (>2,000 bp).

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

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

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

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

Lane Mix Repurification to Remove Linker-Linker Artifacts

A shorter side-product representing linker-linker artifacts is sometimes visible at ~175 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 (≤175 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 31 onwards (p.12).

9. Appendix D: Sequencing*

General

The amount of library loaded onto the flow cell will greatly influence the number of clusters generated. Machine-specific loading instructions can be found in our QuantSeq Frequently Asked Questions (FAQs). QuantSeq FFPE 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 optional, but required in case Unique Molecular Identifier (UMI) read out is desired. The UMI is located within the first 12 nucleotides of Read 2. A schematic representation of those libraries is shown below.
As QuantSeq FFPE library generation is initiated by oligo(dT) priming, a poly(T) sequence will be present after UMI. Therefore, paired-end sequencing with a limited Read 2 (12 to 22 cycles) is required for QuantSeq FFPE libraries.

Dual Indexed Library Sequencing Workflows

The workflow for dual indexed library sequencing differs, depending on the Illumina instrument and flow cell type. Dual indexing can be performed on single-read (SR) and paired-end (PE) flow cells. All HiSeq systems support SR and PE flow cells. NextSeq, MiniSeq, MiSeq, and NovaSeq systems use PE flow cells only, which can also be used in single-read mode. Illumina defines Forward Strand (A) and Reverse Complement (B) Workflows for dual indexing read-out, which refer to the order of Index 2 read-out in relation to Read 2 Resynthesis. For more details about sequencing workflows, please refer to the UDI 12 nt Unique Dual Indexing online FAQs, or contact support@lexogen.com.

The example below shows the sequencing setup for dual indexed QuantSeq FFPE libraries sequenced with the Reverse Complement Workflow (B) on a paired-end flow cell.

EXAMPLE: MiniSeq, iSeq, HiSeq 3000 / 4000 (PE), NovaSeq 6000 (v1.5 chemistry), and NextSeq instruments use the Reverse Complement Workflow (B) with the Multiplexing Index 2 (i5) Sequencing Primer. A minimum of eight cycles with imaging are required for i5 Index read-out, provided the correct UDI set is used for the correct i5 Index read out workflow. 12 nt, 10 nt, or 8 nt can be read out optionally. If QuantSeq preps with UDI Set A (UDI12A_0001-0384) are sequenced on these machines, the i5 Index will be read out as reverse complement. **In this case, all 12 nt of the i5 Index must be read out if error correction shall be applied**.

QuantSeq FFPE libraries:

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

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

Sequencing Primers

Standard Illumina sequencing primers are used for all dual indexed libraries.
The Multiplexing Read 1 Sequencing Primer is always used for Read 1 sequencing, and the Index 1 (i7) Sequencing Primer is always used for Index 1 Read (i7) sequencing. The Index 2 Read (i5) is initiated using different sequencing primers specific to the instrument and flow cell type. For more details, please refer to the User Guide for Lexogen 12 nt Unique Dual Indexing Add-on Kits (198UG445) or contact support@lexogen.com.

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

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

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

Read 2 for QuantSeq FFPE libraries (required for UMI read out):
Read 2 Sequencing Primer (not supplied):
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

NOTE: QuantSeq FFPE libraries are oligo(dT)-primed. Therefore, the poly(T) stretch is located at the beginning of the insert following the UMI in Read 2.

Multiplexing with Other Library Types

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

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

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

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

10. Appendix E: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of QuantSeq FFPE data and is kept as general as possible for integration with your standard pipeline.

Read Orientation

All QuantSeq FFPE 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

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

In addition to the standard error-correction included in the Illumina pipeline, Lexogen's iDemux Tool is freely available on github (https://github.com/Lexogen-Tools) and can be used for both demultiplexing and higher accuracy in error correction. Please contact support@lexogen.com for more information.

Processing Raw Reads

We recommend the use of a general fastq quality control tool such as FastQC or NGS QC Toolkit to examine the quality of the sequencing run. These tools can also identify over-represented sequences, which may optionally be removed from the dataset.

Trimming

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

Alignment

After filtering and trimming, reads can be aligned with a short read aligner to the reference genome. We recommend the use of STAR aligner for mapping QuantSeq data (FWD). The reads may not land in the last exon and span a junction hence splice-aware aligners should be used. Bowtie2, BBMap, or BWA can also be used for mapping against a reference transcriptome.

Annotations and Read Counting

Mapping only the 3′ end of transcripts requires an annotation that covers the 3′ untranslated region (UTR) accurately. Conservative annotations might decrease the power of correct gene quantification after mapping. For some gene annotations it might be an advantage to extend the 3′ UTR annotation further downstream in order to assign the mapped read correctly.

QuantSeq FFPE UMI Data Analysis

QuantSeq FFPE libraries contain 12 nt long Unique Molecular Identifiers (UMI) at the beginning of Read 2. In order to analyze the UMI information, the first 12 nt 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.

Free data analysis on Lexogen's web-based data analysis platform Kangooroo

With each purchased QuantSeq FFPE kit, you receive a voucher code for free data analysis on Kangooroo - Lexogen's web-based data analysis platform (kangooroo.com). The voucher code can be found on a label located on the cardboard above the microtube holder (or one of the holders, if the kit contains more than one). Each provided code allows for the same number of data analysis pipeline runs as the number of reactions included in the library prep kit, including differential expression (DE) analysis. The maximum amount of total data that can be uploaded is 1.5 GB for each sample included in the kit, which is sufficient for most standard QuantSeq FFPE experiments. The voucher code covers the download of the majority of the final results. If you are interested in downloading all data, including pre-processing data like trimmed FASTQ files or BAM files, or if you need to analyze bigger files, please contact us at sales@lexogen.com to purchase additional codes.

11. Appendix F: Automation

ATTENTION: Before starting any new project involving automation, we highly recommend that you reach out to our experts at support@lexogen.com.

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

- Perkin Elmer: Sciclone® / Zephyr®
- Hamilton: Microlab STAR / STARlet / NGS STAR
- Agilent: NGS Workstation (NGS Bravo Option B)
- Beckman Coulter: Biomek FXP , Biomek i5 and Biomek i7
- Eppendorf: EpMotion® 5075
- Opentrons® OT-2

Instrument setups can vary significantly.
If you are interested in adapting automated QuantSeq FWD scripts for QuantSeq FFPE preps, or other liquid handling instruments not listed above, please contact us or check our online FAQs for more information.

12. Appendix G: Revision History

Publication No. / Revision Date	Change	Page
222UG780V0100 Apr. 8, 2024	Initial Release.	

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Associated Products:

022 (Purification Module with Magnetic Beads)
025, 050, 051, 141 (SIRVs Spike-in RNA Variant Control Mixes)
208 (PCR Add-on and Reamplification Kit V2 for Illumina)
235 (DNA Removal Add-on)
236 (SPLIT One-Step FFPE RNA Extraction Kit)

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

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