

The QuantSeq logo consists of the word "QUANT" in green and "SEQ" in dark blue, both in a bold, sans-serif font. A small "TM" trademark symbol is positioned to the upper right of "QUANT".

# QUANT<sup>TM</sup>SEQ

Sequencing that counts

## 3' mRNA-Seq Library Prep Kit FWD HT User Guide

### Catalog Numbers:

- 015 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (FWD) HT including i5 Dual Indexing Add-on Kit (5001-5004))
- 015 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (FWD))
- 016 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (REV) with Custom Sequencing Primer)
- 020 (PCR Add-on Kit for Illumina)
- 022 (Purification Module with Magnetic Beads)
- 025, 050, 051 (Spike-In RNA Variant Controls: SIRV-Set 1, SIRV-Set 2, SIRV-Set 3)
- 026 (QuantSeq-Flex First Strand Synthesis Module for Illumina)
- 028 (QuantSeq-Flex Second Strand Synthesis Module V2 for Illumina)
- 047 (i5 Dual Indexing Add-on Kit for QuantSeq/SENSE (5001-5096))
- 070 (RS-Globin Block, *Homo sapiens*)
- 071 (RS-Globin Block, *Sus scrofa*)
- 080 (Reamplification Add-on Kit for Illumina)
- 081 (UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1))



## ATTENTION: Updated Sequencing Guidelines for Lexogen Libraries

**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, QuantSeq-Flex or SENSE 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).

These recommendations are current as of August 13, 2018. For further information or inquiries please contact [info@lexogen.com](mailto:info@lexogen.com).



## Important recommendations for QuantSeq 3' mRNA Library Prep

### New Changes to the QuantSeq FWD HT Kit (Cat. No. 015.384) and Library Prep Protocol

- The PCR Mix (**PCR** ●) included in the i5 Dual Indexing Add-on Kit, supplied with the QuantSeq FWD HT Kit (015.384), was renamed to Dual PCR Mix (**Dual PCR** ●). This is to clearly distinguish it from the standard PCR Mix (**PCR** ●) provided in the standard QuantSeq FWD (015) Kit. The buffer composition itself has not been changed.
- **The safe stopping point after reverse transcription, i.e., after step 4 has been removed!** – continue directly to RNA removal after reverse transcription of the libraries. Do not place samples on ice and do not store samples at this point. Cooling the samples below room temperature at this point can cause mishybridization.

Best practice handling would be as follows:

1. After the 42 °C incubation is complete, spin down the plate/tubes briefly and place at room temperature.
  2. Immediately add the RNA Removal Solution (RS, thawed at room temperature) to the samples and mix well.
  3. Briefly spin down the plate / tubes at room temperature, then place on the thermocycler to commence the 10 minute incubation at 95 °C (step 6).
- **When storing libraries at -20 °C safe stopping points, ensure the samples are thawed and equilibrated to room temperature before re-starting the protocol.** This is particularly critical before purification steps. Cooler temperatures may negatively affect yields and enhance the presence of unwanted side products.

### Critical Steps for QuantSeq 3' mRNA-Seq Library Prep Kits!

#### First Strand cDNA Synthesis (p.11-12):

1. At step 3 pre-warm the FS2 / E1 mastermix for 2 - 3 minutes at 42 °C – Do not cool the mastermix on ice!
2. Have the RNA / FS1 samples at 42 °C when adding the pre-warmed FS2 / E1 mastermix (step 4) and mix properly when adding. Any drop in temperature at this point can cause mishybridization. Seal the plate or tubes and begin the 42 °C incubation.
3. Spin down the samples at room temperature before and after adding the FS2 / E1 mastermix.

**If step 2 is skipped for low input or degraded samples ( $\leq 10$  ng, or FFPE samples):**

1. Prepare your RNA samples in 5  $\mu$ l volumes.
2. Prepare a mastermix containing 5  $\mu$ l FS1, 9.5  $\mu$ l FS2, and 0.5  $\mu$ l E1, mix well, spin down, and pre-warm at 42 °C on a thermocycler for 2 - 3 minutes.
3. Bring your RNA samples to room temperature while the mastermix is pre-warming.
4. Spin down the pre-warmed FS1 / FS2 / E1 mastermix and add 15  $\mu$ l to each RNA sample. Mix, seal the plate or strip-tubes, spin down briefly at room temperature, and then commence the 42 °C incubation for 15 minutes (or 1 hour for low input RNA ( $\leq 10$  ng)).

**Protocol modifications for low input ( $\leq 10$  ng), FFPE, or low quality / degraded RNA versus standard RNA input:**

Protocol Step	Standard Input ( $> 10$ ng)	Low Input ( $\leq 10$ ng) FFPE / Degraded RNA	Low Input ( $\leq 1$ ng)
Step 2	Incubate for 3 minutes at 85 °C, then cool to 42 °C. Hold samples at 42 °C on the thermocycler.	<b>Skip!</b> Prepare pre-warmed <b>FS1 / FS2 / E1</b> mastermix! Place RNA samples at room temperature.	
Step 3	Prepare <b>FS2 / E1</b> mastermix – pre-warm for 2 - 3 minutes at 42 °C.	Prepare <b>FS1 / FS2 / E1</b> mastermix – pre-warm for 2 - 3 minutes at 42 °C.	
Step 4	Add pre-warmed mastermix to <b>RNA / FS1</b> samples at 42 °C. Incubate for 15 minutes at 42 °C.	Add pre-warmed mastermix to RNA samples at room temperature. <b>OPTIONAL:</b> Increase incubation time to 1 hour at 42 °C.	
Step 6	Incubate for 10 minutes at 95 °C.	Incubate for 10 minutes at 95 °C.	Reduce to 5 minutes at 95 °C.
Step 16	Add 56 $\mu$ l of Purification Solution ( <b>PS</b> ).	Reduce volume of Purification Solution ( <b>PS</b> ) to 48 $\mu$ l.	
Step 24	The qPCR assay is strongly recommended when processing samples with: <ul style="list-style-type: none"> <li>• Variable input amounts</li> <li>• Variable RNA quality</li> <li>• Different or new sample types (e.g., species, tissue, cell type)</li> </ul>	The qPCR assay is strongly recommended for all low input, FFPE / degraded RNA library preps, to prevent over- or undercycling of the libraries.	
Step 29	<b>Single-indexed libraries:</b> Add 30 $\mu$ l of Purification Beads ( <b>PB</b> ).	<b>Single-indexed libraries:</b> Reduce volume of Purification Beads ( <b>PB</b> ) to 27 $\mu$ l.	
Step 30	<b>Dual-indexed libraries:</b> Add 35 $\mu$ l of Purification Beads ( <b>PB</b> ).	<b>Dual-indexed libraries:</b> Reduce volume of Purification Beads ( <b>PB</b> ) to 31.5 $\mu$ l.	

**ATTENTION:** The QuantSeq 3' mRNA-Seq Library Prep protocol has been upgraded in February 2017. Changes include new arrangement and renaming of the barcode plate to improve the nucleotide balance and streamlining of the protocol (e.g., removal of RS2 solution). For more details please check the Revision History at the end of this User Guide or contact us under [support@lexogen.com](mailto:support@lexogen.com) or +43 1 3451212 41.

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### **LITERATURE CITATION**

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

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# 1. Overview

Lexogen's QuantSeq kit FWD HT provides a high-throughput library preparation protocol designed to generate Illumina-compatible libraries from polyadenylated RNA within 4.5 hours. The QuantSeq protocol generates only one fragment per transcript, resulting in extremely accurate gene expression values. The sequences obtained are close to the 3' end of the transcripts.

QuantSeq FWD HT (Cat. No. 015.384) is a high-throughput version (384 preps) with optional dual indexing to prevent sample mix-ups and to increase the number of available indices. The Read 1 linker sequence is located at the second strand synthesis primer, hence NGS reads are generated towards the poly(A) tail and directly correspond to the mRNA sequence. To pinpoint the exact 3' end longer reads may be required. Although paired-end sequencing is possible, we do not recommend it for QuantSeq FWD HT (Cat. No. 015.384). Read 2 would start with the poly(T) stretch, and as a result of sequencing through the homopolymer stretch the quality of Read 2 would be very low.

QuantSeq FWD HT maintains strand-specificity and allows mapping of reads to their corresponding strand on the genome, enabling the discovery and quantification of antisense transcripts and overlapping genes. The kit includes magnetic beads for the purification steps and hence is compatible with automation. Multiplexing of libraries can be carried out using up to 96 i7 indices (provided in a 96-well plate) and four perfectly balanced i5 indices (provided in microtubes), resulting in 384 unique index combinations.

QuantSeq uses total RNA as input, hence no prior poly(A) enrichment or rRNA depletion is required. Information regarding input RNA requirements can be found in Appendix A, p.22.

Library generation is initiated by oligo(dT) priming. The primer already contains Illumina-compatible linker sequences. After first strand synthesis the RNA is removed and second strand synthesis is initiated by random priming and a DNA polymerase. The random primer also contains Illumina-compatible linker sequences. No purification is required between first and second strand synthesis. The insert size is optimized for shorter reads (SR50, SR75, SR100).

Second strand synthesis is followed by a magnetic bead-based purification step. The library is then amplified, introducing the sequences required for cluster generation (see Appendix L, p.41 for a schematic representation of the finished library). i5 and i7 multiplexing indices are included in the QuantSeq FWD HT kit (Cat. No. 015.384) and are introduced during this step. For index sequences refer to Appendix K, p.38. More details on index read-out on various Illumina platforms can be found in Appendix L, p.41.

Library quantification can be performed with standard protocols (see Appendix G, p.30). Data can be analyzed with a number of standard bioinformatics pipelines. Special considerations for the analysis of QuantSeq data, such as read orientation, are presented in Appendix M, p.45. A QuantSeq bioinformatics pipeline has been integrated on the Bluebee genomics analysis platform and each purchased QuantSeq Kit includes a code for free data analysis on the Bluebee platform. For more details visit our webpage at [www.lexogen.com](http://www.lexogen.com).

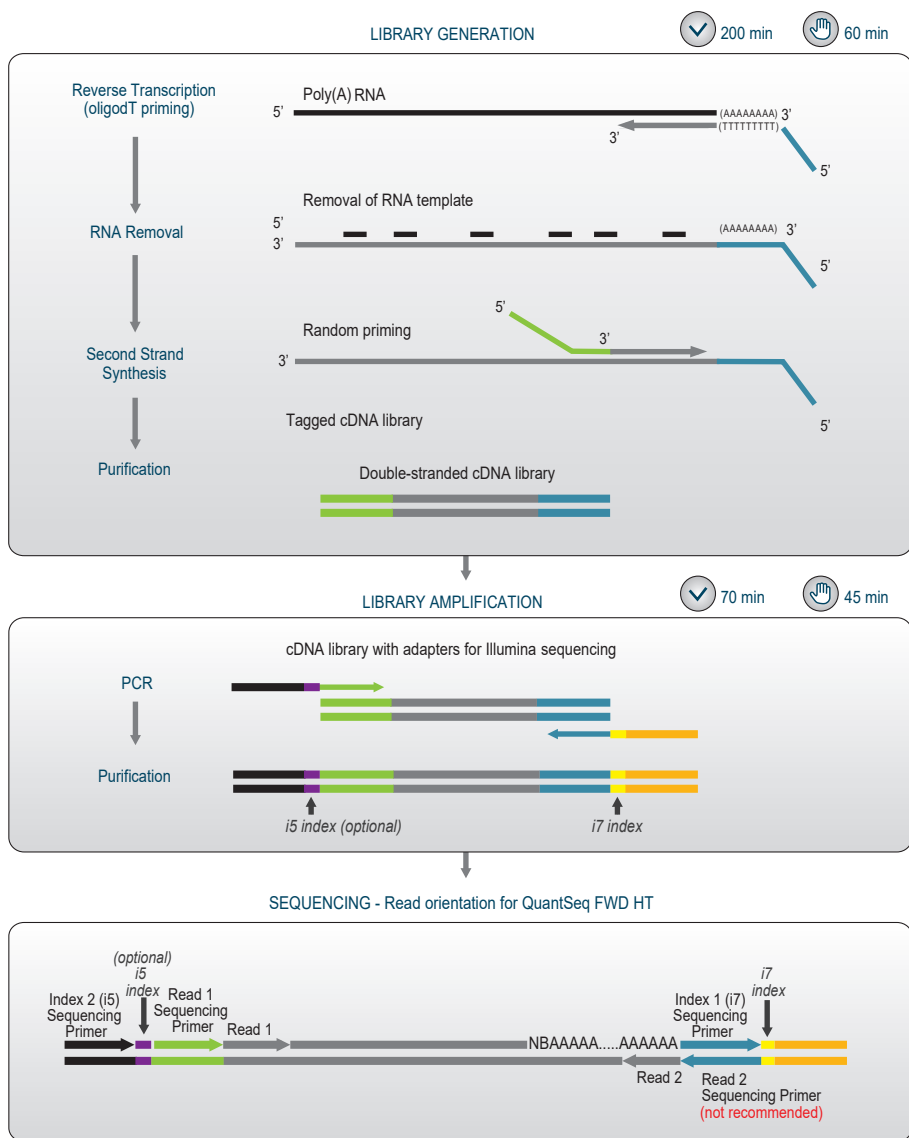


Figure 1. Schematic overview of the QuantSeq FWD HT library preparation workflow (Cat. No. 015.384). Illumina P5 adapters (Read 1) are shown in green and Illumina P7 adapters (Read 2) are shown in blue. Read 1 reflects the mRNA sequence. Paired-end sequencing is not recommended for QuantSeq FWD HT (Cat.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



## 2. Kit Components and Storage Conditions

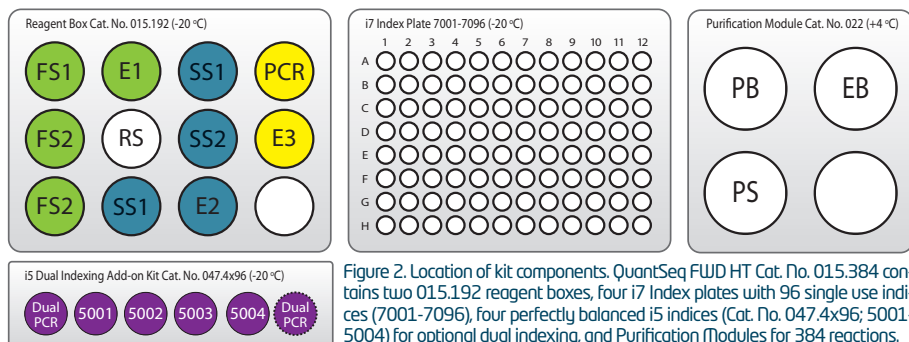


Figure 2. Location of kit components. QuantSeq FWD HT Cat. No. 015.384 contains two 015.192 reagent boxes, four i7 Index plates with 96 single use indices (7001-7096), four perfectly balanced i5 indices (Cat. No. 047.4x96; 5001-5004) for optional dual indexing, and Purification Modules for 384 reactions.

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

\*including 10 % surplus

Upon receiving the QuantSeq kit, store the Purification Module (Cat. No. 022.96), containing **PB**, **PS**, and **EB** at +4 °C and the rest of the kit in a -20 °C freezer. **REMARK:** **EB** can be stored either at +4 °C or -20 °C. Before use, check the contents of **PS**. If a precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

## 3. User-Supplied Consumables and Equipment

Check to ensure that you have all of the necessary material and equipment before beginning the library preparation. All reagents, equipment, and labware must be free of nucleases and nucleic acid contamination.

### Reagents / Solutions

- 80 % fresh ethanol (for washing of Purification Beads, **PB**).
- Optional: SYBR Green I (Sigma-Aldrich, Cat. No. S9430), 10,000x in DMSO for qPCR.

### Equipment

- Magnetic plate e.g., 96S Super Magnet Plate, article# A001322 from Alpaqua.
- Benchtop centrifuge (3,000 x g, rotor compatible with 96-well plates).
- Calibrated single-channel pipettes for handling 1 µl to 1,000 µl volumes.
- Calibrated multi-channel pipettes for handling 1 µl to 200 µl volumes.
- Thermocycler.
- UV-spectrophotometer to quantify RNA.
- Ice bath or ice box, ice pellets, benchtop cooler (-20 °C for enzymes).

### Labware

- Suitable certified ribonuclease-free low binding pipette tips (pipette tips with aerosol barriers recommended).
- 1.5 ml reaction tubes, low binding, certified ribonuclease-free.
- 200 µl PCR tubes or 96-well plates and caps or sealing foil.
- Vortex mixer.

### Optional Equipment

- Automated microfluidic electrophoresis station (e.g., Agilent Technologies 2100 Bioanalyzer).
- qPCR machine and library standards (for library quantification).
- Benchtop fluorometer and appropriate assays (for RNA quality control and library quantification).
- Agarose gels, dyes, and electrophoresis rig (for RNA quality control).

The complete set of material, reagents, and labware necessary for RNA extraction and quality control is not listed. Consult Appendix A and B, p.22 and p.24 ,for more information on RNA quality.

Consult Appendix G, p.30 for information on library quantification methods.

## 4. Guidelines

### RNA Handling

- RNases are ubiquitous, and special care should be taken throughout the procedure to avoid RNase contamination.
- Use commercial ribonuclease inhibitors (i.e., RNasin, Promega Corp.) to maintain RNA integrity when storing samples.
- Use a sterile and RNase-free workstation or laminar flow hood if available. Please note that RNases may still be present on sterile surfaces, and that autoclaving does not completely eliminate RNase contamination. Well before starting a library preparation, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) as per the manufacturer's instructions. **ATTENTION:** Do not forget to rinse off any RNaseZap residue with RNase-free water after usage! Residues of RNaseZap may damage the RNA.
- Protect all reagents and your RNA samples from RNases on your skin by wearing a clean lab coat and fresh gloves. Change gloves after making contact with equipment or surfaces outside of the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

### Bead Handling

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

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

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

## General

- Unless explicitly mentioned, all steps including centrifugations, should be carried out at room temperature (RT) between 20 °C and 25 °C. Results may be negatively impacted if the protocol is performed at temperatures outside of this range. While reaction set-up is often performed at RT, incubation temperatures are explicitly defined, and must be strictly adhered to.
- To further increase reproducibility and to avoid cross contamination a centrifugation step should be performed after incubations at elevated temperatures and before removing the sealing foil from PCR plates (e.g., step 2).
- Ensure that adequate volumes of all reagents and the necessary equipment are available before beginning the protocol.
- Perform all pipetting steps with calibrated pipettes, and always use fresh tips. Pipette carefully to avoid foaming as some solutions contain detergents.
- Thaw all necessary buffers at room temperature or as indicated in the preparation tables at the beginning of each step of the detailed protocol. Mix reagents well by vortexing or pipetting repeatedly and centrifuge briefly with a benchtop centrifuge to collect contents before use.
- Keep Enzyme Mixes at -20 °C until just before use or store in a -20 °C benchtop cooler.
- Steps requiring a thermocycler have been tested with a maximum ramp speed of 5 °C/sec before denaturation and extension, and 2.5 °C/sec during primer annealing. While these ramp speeds are typical for most modern thermocyclers, some models can exceed these

rates, and ramp speed may need to be decreased to ensure efficient annealing. Ramp speeds may be reduced even further in some steps of the protocol to ensure better hybridization. Preheat lid to 105 °C, in case this has to be adjusted manually.

- When mixing by pipetting, set the pipette to a larger volume. For example after adding 5 µl in steps 5 and 10 use a pipette set to 15 µl or 30 µl, respectively, to ensure proper mixing.

## Pipetting and Handling of (Viscous) Solutions

- Enzyme Mixes, **SS1** ●, **PB**, and **PS** are viscous solutions which require care to pipette accurately. Quickly spin down the tubes to collect all liquid at the bottom of the tube. Be sure to pipette slowly and check the graduation marks on your pipette tips when removing an aliquot.
- When drawing up liquid, the tip should be dipped 3 to 5 mm below the surface of the liquid, always at a 90 degree angle. Do not dip the tip in any further as viscous solutions tend to stick to the outside of the pipette tip.
- Any residual liquid adhering to the tip should be removed by sliding the tip up the wall or edge of the tube from which the liquid was taken. Spin down the tube afterwards again to ensure that all liquid is collected at the bottom of the tube for further storage.
- When dispensing, the pipette should be held at a 45 degree angle, and the tip placed against the side of the receiving vessel.
- When pipetting liquids from bottles, take special care that only the sterile pipette tip touches the bottle opening to prevent introducing RNases or other contaminants. Tips are sterile, whereas the pipette itself is not. If necessary, tilt the bottle to bring the liquid closer to the opening and facilitate pipetting.

## Preparation of Mastermixes and Pipetting with Multi-Channel Pipettes

In steps 3, 9, and 25 of the QuantSeq HT protocol, mastermixes of enzymes and reaction buffers should be prepared. When preparing mastermixes and when using multi-channel pipettes always include a 10% surplus per reaction in order to have enough solution available for all reactions.

**EXAMPLE:** Step 3 for 24 preps: use 250.8 µl **FS2** ● (= 9.5 µl x 24 rxn x 1.1)  
+ 13.2 µl **E1** ● (= 0.5 µl x 24 rxn x 1.1)

resulting in a total of 264 µl, which is sufficient for multi-channel pipetting.

All reagents of the QuantSeq HT kit include a 10 % surplus.

## Automation

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

# 5. Detailed Protocol

## 5.1 Library Generation

### Preparation

First Strand cDNA Synthesis	RNA Removal	Second Strand Synthesis	Purification
<b>FS1</b> ● – thawed at RT <b>FS2</b> ● – thawed at RT <b>E1</b> ● – keep on ice or at -20 °C	<b>RS</b> ○ – thawed at RT	<b>SS1</b> ● – thawed at 37 °C <b>SS2</b> ● – thawed at RT <b>E2</b> ● – keep on ice or at -20 °C	<b>PB</b> – stored at +4 °C <b>PS</b> – stored at +4 °C <b>80 % EtOH</b> – provided by user <b>prepare fresh!</b> <b>EB</b> – stored at +4 °C
85 °C, 3 min 42 °C, 15 min	95 °C, 10 min cool down to 25 °C	98 °C, 1 min, then cool to 25 °C (0.5 °C/sec) 25 °C, 30 min 25 °C, 15 min	Equilibrate all reagents to room temperature for 30 minutes prior to use.

### First Strand cDNA Synthesis - Reverse Transcription

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

**ATTENTION:** The minimum recommended input amount for QuantSeq FWD HT Library Prep is 100 pg. When using input amounts ≤1ng we recommend including a no-input control.

**NOTE:** Protocol modifications are recommended for low input (≤10 ng), low quality, and FFPE RNA samples. These are indicated as **"REMARK"** in the respective protocol steps (see also Appendix C, p.26).

Mix 100 pg - 2 µg of total RNA in a volume of 5 µl, with 5 µl First Strand cDNA Synthesis Mix 1 (**FS1** ●) in a PCR plate or 8-well strip tubes. If necessary, adjust the total volume to 10 µl with RNase-free water. Mix well by pipetting. Ensure the plate or PCR strips / tubes are tightly sealed, and spin down to collect the liquid at the bottom of the wells.

**REMARK:** For low input (≤10 ng), FFPE, or low quality RNA, do not add **FS1** ● to the RNA. Instead place the RNA samples at room temperature while preparing the master-mix (see below, and Appendix C, p.26 and Appendix D, p.27).

Denature the RNA / **FS1** mix for 3 minutes at 85 °C in a thermocycler and then cool down to 42 °C. **ATTENTION:** Leave the reactions at 42 °C until step 4. **REMARK:** Skip this step for low input amounts (≤10 ng), low quality, or FFPE RNA.

3

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

4

Quickly spin down the denatured RNA / **FS1** samples from step 2 at room temperature to make sure all liquid is collected at the bottom of the wells. Place the samples back onto the thermocycler at 42 °C and carefully remove the sealing foil / tube caps. Add 10 µl of the **FS2** / **E1** mastermix to each reaction, mix well, and seal the plate / tubes. If necessary, quickly spin down the liquid at room temperature. Incubate the reactions for 15 minutes at 42 °C. **REMARK:** If step 2 is skipped, add 15 µl of the pre-warmed **FS1** / **FS2** / **E1** mastermix to each 5 µl RNA sample. Seal the plate / tubes and mix with gentle vortexing. Quickly spin down at room temperature and incubate the reactions for 15 minutes at 42 °C. **OPTIONAL:** For low input (≤10 ng), FFPE, or low quality RNA, extend the incubation time to 1 hour at 42 °C. **ATTENTION:** Proceed immediately to the RNA removal step! Do not cool the samples below room temperature after reverse transcription!

## RNA Removal

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

**OPTIONAL:** At step 5, the Globin Block Modules for QuantSeq (RS-Globin Block, *Homo sapiens* (**RS-GBHs** ●), Cat. No. 070; and RS-Globin Block, *Sus scrofa* (**RS-GBSs** ●), Cat. No. 071) can be used instead of the standard RNA Removal Solution (**RS O**) (see Appendix I, p.34). The use of RS-Globin Block prevents the generation of amplifiable library fragments from globin mRNAs, when preparing QuantSeq libraries from blood total RNA.

5

Add 5 µl RNA Removal Solution (**RS O**) directly to the first strand cDNA synthesis reaction. Mix well and reseal the plate using a fresh foil. **REMARK:** Use a pipette set to 15 µl for efficient mixing.

6

Incubate 10 minutes at 95 °C, then cool down to 25 °C. Spin down the plate at room temperature and carefully remove the sealing foil. **REMARK:** Reduce this step for RNA inputs ≤1 ng total RNA, to 5 minutes at 95 °C (see Appendix C, p.26).

## Second Strand Synthesis

During this step the library is converted to dsDNA. Second strand synthesis is initiated by a random primer containing an Illumina-compatible linker sequence at its 5' end. A reverse complement prevents the linker sequence from taking part in the hybridization.

**ATTENTION:** Second Strand Synthesis Mix 1 (**SS1** ●) is a viscous solution and needs to be mixed thoroughly before use. Thaw at 37 °C for 5 minutes. If a precipitate is visible, incubate for a further 5 minutes at 37 °C and mix until buffer components dissolve completely.

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

**OPTIONAL:** At step 7 the UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1, Cat. No. 081) may be used to include Unique Molecular Identifiers (UMIs) in QuantSeq FWD libraries. The UMI Second Strand Synthesis Mix (**USS** ●) replaces the Second Strand Synthesis Mix 1 (**SS1** ●) from the standard QuantSeq FWD Kit (Cat. No. 015) (see Appendix J, p.36).

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7 Add 10 µl Second Strand Synthesis Mix 1 (**SS1** ●) to the reaction. Mix well by pipetting, and seal the plate. **REMARK:** Use a pipette set to 30 µl for efficient mixing.

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8 Incubate the plate for 1 minute at 98 °C in a thermocycler, and slowly cool down to 25 °C by setting the ramp speed to 10 % (0.5 °C/second). Incubate the reaction for 30 minutes at 25 °C. Quickly spin down the plate at room temperature before removing the sealing foil.


---

9 Prepare a mastermix containing 4 µl Second Strand Synthesis Mix 2 (**SS2** ●) and 1 µl Enzyme Mix 2 (**E2** ●). Mix well.

---

10 Add 5 µl of the **SS2** / **E2** mastermix per reaction. Mix well. **REMARK:** Use a pipette set to 30 µl for efficient mixing.

---

11 Incubate the reaction for 15 minutes at 25 °C.  Safe stopping point. Libraries can be stored at -20 °C at this point.


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## Purification

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

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

- 12 Add 16 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature.
- 13 Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear (depends on the strength of your magnet).
- 14 Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
- 15 Add 40 µl of Elution Buffer (**EB**), remove the plate from the magnet, and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
- 16 Add 56 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly, and incubate for 5 minutes at room temperature. **REMARK:** For low input (≤10 ng), FFPE, or low quality RNA, add only 48 µl **PS** (see Appendices C and D, p.26-27).
- 17 Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
- 18 Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
- 19 Add 120 µl of 80 % EtOH, and incubate the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.
- 20 Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely as traces of ethanol can inhibit subsequent PCR reactions.
- 21 Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.
- 22 Add 20 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
- 23 Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
- 24 Transfer 17 µl of the clear supernatant into a fresh PCR plate. Make sure not to transfer any beads.  Safe stopping point. Libraries can be stored at -20 °C at this point. See Appendix E, p.28 for qPCR options.

## 5.2. Library Amplification

The library is amplified to add the complete adapter sequences required for cluster generation and to generate sufficient material for quality control and sequencing. For single indexing (i7 index only), follow protocol 5.2.1 (p.15-17). For dual indexing (i5 and i7 indices), follow protocol 5.2.2 (p.17-19). PCR and post-PCR purification volumes differ between the two indexing options and are discussed in detail in the respective subsections.

**ATTENTION:** PCR cycle numbers may differ depending on the RNA used. At this point, **we strongly recommend performing a qPCR assay to determine the optimal PCR cycle number** for your particular RNA sample type. The PCR Add-on Kit for Illumina (Cat. No. 020) provides the additional reagents required. For details of the qPCR assay please see Appendix E, p.28. Example PCR cycle numbers for RNA from a variety of organisms and tissues as provided in Appendix B, p.24, should be taken as a guideline only.

### 5.2.1 Single Indexing (i7 only)

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

### Preparation

PCR		Purification
<b>PCR</b> ●	– thawed at RT	<b>PB</b> – stored at +4 °C
<b>E3</b> ●	– keep on ice or at -20 °C	<b>PS</b> – stored at +4 °C
<b>7001-7096 (i7 Indices)</b>	– thawed at RT; in 96-well plate; <b>spin down before opening!</b>	<b>80 % EtOH</b> – provided by user; <b>prepare fresh!</b>
		<b>EB</b> – stored at +4 °C
Thermocycler	98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞	Equilibrate all reagents to room temperature for 30 minutes prior to use.
	<div>11 -22x see Appendix B, p.24 see Appendix D, p.27</div>	

### PCR

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

25


Prepare a mastermix containing 7 µl of PCR Mix (**PCR** ●) and 1 µl Enzyme Mix 3 (**E3** ●) per reaction.

26

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

Add 5 µl of the respective i7 index (**7001-7096**, in 96-well plate). Mix well by pipetting. Seal the PCR plate and quickly spin down to make sure all liquid is collected at the bottom of the well. **ATTENTION:** Spin down the i7 Index Plate before opening! Visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired indices. Avoid cross contamination! Reseal opened wells of the i7 Index Plate after usage to prevent cross contamination! **NOTE:** Each well of the i7 Index Plate is intended for single use only!

27

Conduct 11 - 22 cycles of PCR (see recommendations in Appendices B and D, p.24, and p.27), or determine by qPCR, see Appendix E, p.28) with: Initial denaturation at 98 °C for 30 seconds, 11 - 22 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, hold at 10 °C.  Safe stopping point. Libraries can be stored at -20 °C at this point.

28

## Purification

The finished library is purified from PCR components that can interfere with quantification. The Purification Beads (**PB**) must be properly resuspended before adding them to the reaction!

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

29

Add 30 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature. **REMARK:** For low input (≤10 ng), FFPE, or low quality RNA, add only 27 µl Purification Beads (**PB**) (see Appendix C and D, p.26-27).

30

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

31

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

32

Add 30 µl of Elution Buffer (**EB**), remove the plate from the magnet, and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.

33

Add 30 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly, and incubate for 5 minutes at room temperature.

34


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

35

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

36

Add 120 µl of 80 % EtOH, and incubate the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.

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

## 5.2.2 Dual Indexing (i7 and i5)

The following protocol replaces the single indexing PCR and post-PCR purification protocol described in steps 25 - 41 on pages 15-17. If a qPCR is intended to determine the exact cycle number for endpoint PCR, use the PCR Add-on Kit (Cat. No. 020, see also Appendix E, p.28).

**ATTENTION:** For dual indexing use the **Dual PCR Mix (Dual PCR ●)**. **DO NOT USE** the yellow-cap **PCR Mix (PCR ●)**, as this contains a non-indexed P5 primer and will result in a loss of i5 indexing.

## Preparation

PCR		Purification (Cat. No. 022)
<div>From i5 Dual Indexing Add-on Kit: Dual PCR ● 5001 - 5004 ● or 5001 - 5096 (i5 Index Plate) – thawed at RT</div> <div>From standard library prep kit: 7001 - 7096 (i7 Index Plate) E3 ●</div> <div>– thawed at RT – keep on ice or at -20 °C</div> <div>spin down before opening!</div>		<div>from standard library prep kits: PB – stored at +4°C PS – stored at +4°C 80 % EtOH – provided by user prepare fresh! EB – stored at +4°C</div>
Thermocycler	<div>98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞</div> <div>11- 27x see Appendix B, p.24 see Appendix D, p.27</div>	Equilibrate all reagents to room temperature for 30 minutes prior to use.

## PCR

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

25 Prepare a mastermix containing 7 µl Dual PCR Mix (**Dual PCR** ●) and 1 µl Enzyme Mix 3 (**E3** ●). **ATTENTION:** Do not use the yellow-cap PCR Mix (**PCR** ●) if dual indexing is intended!

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

27 Add 5 µl of the respective i5 Index Primer (**5001 - 5004** ●, in microtubes, or **5001 - 5096**, in 96-well plate, from the i5 Unique Dual Indexing Add-on Kit). **ATTENTION:** Spin down the i5 tubes / Index Plate before opening! Pierce or cut open the sealing foil of the wells containing the desired barcodes. Avoid cross contamination! Reseal opened wells of the barcode plate after use to prevent cross contamination!

28 Add 5 µl of the respective i7 Index Primer (**7001 - 7096**, in 96-well plate, supplied with the standard kits). **REMARK:** Ensure the total PCR volume is 35 µl. If necessary, adjust the volume with Elution Buffer (**EB**) or molecular biology-grade water. Mix well by pipetting. Seal the PCR plate and quickly spin down to make sure all liquid is collected at the bottom of the well. **ATTENTION:** Spin down the i7 Index Plate before opening! Pierce or cut open the sealing foil of the wells containing the desired barcodes. Avoid cross contamination! Reseal opened wells of the barcode plate after use to prevent cross contamination! **NOTE:** Each well of the i7 Index Plate is intended for single use only!

29 Conduct 11- 22 cycles of PCR (see recommendations in Appendices B and D, p.24, and p.27), or determine by qPCR, see Appendix E, p.28) with: Initial denaturation at 98 °C for 30 seconds, 11 - 22 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, hold at 10 °C. 📌 Safe stopping point. Libraries can be stored at -20 °C at this point.

## Purification

The finished library is purified from PCR components that can interfere with quantification. The Purification Beads (**PB**) may have settled and must be properly resuspended before adding them to the reaction.

**ATTENTION:** If PCR products were stored at -20 °C, ensure these are thawed and equilibrated to room temperature before Purification Beads (**PB**) are added.

30 Add 35 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature. **REMARK:** For low input (≤10 ng), low quality, or FFPE RNA, add only 31.5 µl **PB** (see Appendices C and D, p.26-27).

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

---

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

---

- 33 Add 30 µl of Elution Buffer (**EB**), remove the plate from the magnet, and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.

---

- 34 Add 30 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly, and incubate for 5 minutes at room temperature.

---

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

---

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

---

- 37 Add 120 µl of 80 % EtOH, and incubate the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.

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- 38 Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.

---

- 39 Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.

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- 40 Add 20 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet, and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.


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

---

- 42 Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Make sure not to transfer any beads.

---

- 43 At this point, the libraries are finished and ready for for quality control (Appendix G, p.30), pooling (for multiplexing see Appendix K p.38), and cluster generation.  Safe stopping point. Libraries can be stored at -20 °C at this point.

## 6. Short Procedure

### 6.1 Single Indexing (i7 only)

**ATTENTION:** Spin down solutions before opening tubes or plates! **200 min** **Library Generation**

<input type="checkbox"/>	Mix 5 µl RNA and 5 µl <b>FS1</b> ●.	First Strand cDNA Synthesis
<input type="checkbox"/>	Incubate for 3 min at 85 °C, then cool to 42 °C. Skip this step for low input (≤10 ng), FFPE, or low quality RNA. <b>ATTENTION:</b> Keep samples on thermocycler at 42 °C.	
<input type="checkbox"/>	Prepare a mastermix with 9.5 µl <b>FS2</b> ● and 0.5 µl <b>E1</b> ● per reaction, mix well and pre-warm for 2 - 3 min at 42 °C. Do not cool the mastermix on ice!	
<input type="checkbox"/>	Add 10 µl <b>FS2</b> / <b>E1</b> mix per reaction, mix well.	
<input type="checkbox"/>	Incubate for 15 min at 42 °C. <b>OPTIONAL:</b> May be increased to 1 hr for low input (≤10 ng), FFPE, or low quality RNA.	
<input type="checkbox"/>	Add 5 µl <b>RS O</b> , mix well.	RNA Removal
<input type="checkbox"/>	Incubate 10 min at 95 °C, then cool to 25 °C. Reduce to 5 min at 95 °C for ≤1 ng RNA input.	
<input type="checkbox"/>	Add 10 µl <b>SS1</b> ●, mix well.	Second Strand Synthesis
<input type="checkbox"/>	Incubate 1 min at 98 °C, slowly ramp down to 25 °C (0.5 °C / sec).	
<input type="checkbox"/>	Incubate 30 min at 25 °C.	
<input type="checkbox"/>	Prepare a mastermix with 4 µl <b>SS2</b> ● and 1 µl <b>E2</b> ● per reaction, mix well.	
<input type="checkbox"/>	Add 5 µl <b>SS2</b> / <b>E2</b> mix per reaction, mix well.	
<input type="checkbox"/>	Incubate 15 min at 25 °C. 🛑 Safe stopping point.	
<input type="checkbox"/>	Add 16 µl <b>PB</b> per reaction, mix well, incubate 5 min.	Purification
<input type="checkbox"/>	Place on magnet for 2 - 5 min, remove, and discard supernatant.	
<input type="checkbox"/>	Add 40 µl <b>EB</b> , remove from magnet, mix well, incubate 2 min at RT.	
<input type="checkbox"/>	Add 56 µl <b>PS</b> (or 48 µl <b>PS</b> for low input (≤10 ng), FFPE, or low quality RNA), mix well, incubate 5 min at RT.	
<input type="checkbox"/>	Place on magnet for 2 - 5 min, remove, and discard supernatant.	
<input type="checkbox"/>	Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.	
<input type="checkbox"/>	Air dry beads for 5 - 10 min. <b>ATTENTION:</b> Do not let the beads dry too long!	
<input type="checkbox"/>	Add 20 µl <b>EB</b> , remove from magnet, mix well, incubate 2 min at RT.	
<input type="checkbox"/>	Place on magnet for 2 - 5 min, transfer 17 µl of the supernatant into a fresh PCR plate. 🛑 Safe stopping point.	

**70 min** **Library Amplification**

<input type="checkbox"/>	Prepare a mastermix with 7 µl <b>PCR</b> ● and 1 µl <b>E3</b> ● per reaction, mix well.	PCR
<input type="checkbox"/>	Add 8 µl <b>PCR</b> / <b>E3</b> premix to 17 µl of each purified library.	
<input type="checkbox"/>	Add 5 µl i7 primer ( <b>7001-7096</b> , from the 96-well plate), mix well. <b>ATTENTION:</b> Reseal opened index wells after usage!	
<input type="checkbox"/>	<div> <div> <div>PCR: 98 °C, 30 sec</div> <div> <div>98 °C, 10 sec</div> <div>65 °C, 20 sec</div> <div>72 °C, 30 sec</div> <div>72 °C, 1 min</div> <div>10 °C, ∞, 🛑 Safe stopping point.</div> </div> </div> <div> <div>11 - 22x</div> <div>(see p.24-27)</div> </div> </div> <b>ATTENTION: Increase cycle number for low input RNA and samples with low mRNA content! (see p.24-27)</b>	
<input type="checkbox"/>	Add 30 µl <b>PB</b> (or 27 µl <b>PB</b> for low input (≤10 ng), FFPE, or low quality RNA) per reaction, mix well, incubate 5 min.	
<input type="checkbox"/>	Place on magnet for 2 - 5 min, remove, and discard supernatant.	Purification
<input type="checkbox"/>	Add 30 µl <b>EB</b> , remove from magnet, mix well, incubate 2 min at RT.	
<input type="checkbox"/>	Add 30 µl <b>PS</b> , mix well, incubate 5 min at RT.	
<input type="checkbox"/>	Place on magnet for 2 - 5 min, remove and discard supernatant.	
<input type="checkbox"/>	Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.	
<input type="checkbox"/>	Air dry beads for 5 - 10 min. <b>ATTENTION:</b> Do not let the beads dry too long!	
<input type="checkbox"/>	Add 20 µl <b>EB</b> , remove from magnet, mix well, incubate 2 min at RT.	
<input type="checkbox"/>	Place on magnet for 2 - 5 min, transfer 15 - 17 µl of the supernatant into a fresh PCR plate. 🛑 Safe stopping point.	

# 6.2 Dual Indexing (i7 and i5)

**ATTENTION:** Spin down solutions before opening tubes or plates!

200 min

Library Generation

<input type="checkbox"/> Mix 5 µl RNA and 5 µl <b>FS1</b> ●.	First Strand cDNA Synthesis
<input type="checkbox"/> Incubate for 3 min at 85 °C, then cool to 42 °C. Skip this step for low input (≤10 ng), FFPE, or low quality RNA. <b>ATTENTION:</b> Keep samples on thermocycler at 42 °C.	
<input type="checkbox"/> Prepare a mastermix with 9.5 µl <b>FS2</b> ● and 0.5 µl <b>E1</b> ● per reaction, mix well and pre-warm for 2 - 3 min at 42 °C. Do not cool the mastermix on ice!	
<input type="checkbox"/> Add 10 µl <b>FS2</b> / <b>E1</b> mix per reaction, mix well.	
<input type="checkbox"/> Incubate for 15 min at 42 °C. <b>OPTIONAL:</b> May be increased to 1 hr for low input (≤10 ng), FFPE, or low quality RNA.	RNA Removal
<input type="checkbox"/> Add 5 µl <b>RS</b> ○, mix well.	
<input type="checkbox"/> Incubate 10 min at 95 °C, then cool to 25 °C. Reduce to 5 min at 95 °C for ≤1 ng RNA input.	Second Strand Synthesis
<input type="checkbox"/> Add 10 µl <b>SS1</b> ●, mix well.	
<input type="checkbox"/> Incubate 1 min at 98 °C, slowly ramp down to 25 °C (0.5 °C / sec).	
<input type="checkbox"/> Incubate 30 min at 25 °C.	
<input type="checkbox"/> Prepare a mastermix with 4 µl <b>SS2</b> ● and 1 µl <b>E2</b> ● per reaction, mix well.	
<input type="checkbox"/> Add 5 µl <b>SS2</b> / <b>E2</b> mix per reaction, mix well.	
<input type="checkbox"/> Incubate 15 min at 25 °C. 🛑 Safe stopping point.	Purification
<input type="checkbox"/> Add 16 µl <b>PB</b> per reaction, mix well, incubate 5 min.	
<input type="checkbox"/> Place on magnet for 2 - 5 min, remove, and discard supernatant.	
<input type="checkbox"/> Add 40 µl <b>EB</b> , remove from magnet, mix well, incubate 2 min at RT.	
<input type="checkbox"/> Add 56 µl <b>PS</b> (or 48 µl <b>PS</b> for low input (≤10 ng), FFPE, or low quality RNA), mix well, incubate 5 min at RT.	
<input type="checkbox"/> Place on magnet for 2 - 5 min, remove, and discard supernatant.	
<input type="checkbox"/> Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.	
<input type="checkbox"/> Air dry beads for 5 - 10 min. <b>ATTENTION:</b> Do not let the beads dry too long!	
<input type="checkbox"/> Add 20 µl <b>EB</b> , remove from magnet, mix well, incubate 2 min at RT.	
<input type="checkbox"/> Place on magnet for 2 - 5 min, transfer 17 µl of the supernatant into a fresh PCR plate. 🛑 Safe stopping point.	

70 min

Library Amplification

<input type="checkbox"/>	Prepare a mastermix with 7 µl <b>Dual PCR</b> ● and 1 µl <b>E3</b> ● per reaction, mix well.		
<input type="checkbox"/>	Add 8 µl <b>Dual PCR</b> / <b>E3</b> premix to 17 µl of each purified library.		
<input type="checkbox"/>	Add 5 µl <b>i5</b> (in microtubes or 96-well i5 Index Plate, from the i5 Dual Indexing Add-on Kit) for each reaction, mix well.		
<input type="checkbox"/>	Add 5 µl <b>i7</b> (from the 96-well i7 Index Plate) for each reaction, mix well. <b>ATTENTION:</b> Reseal opened index wells after usage!		
<input type="checkbox"/>	PCR: 98 °C, 30 sec	PCR	
	<div><div><div>98 °C, 10 sec</div><div>65 °C, 20 sec</div><div>72 °C, 30 sec</div><div>72 °C, 1 min</div><div>10 °C, ∞. 🛑 Safe stopping point.</div></div><div>}</div><div>11 - 22x (see p.24-27)</div></div>		<b>ATTENTION: Increase cycle number for low input RNA and samples with low mRNA content! (see p.24-27)</b>
<input type="checkbox"/>	Add 35 µl <b>PB</b> (or 31.5 µl <b>PB</b> for low input (≤10 ng), FFPE, or low quality RNA) per reaction, mix well, incubate 5 min.	Purification	
<input type="checkbox"/>	Place on magnet for 2 - 5 min, remove, and discard supernatant.		
<input type="checkbox"/>	Add 30 µl <b>EB</b> , remove from magnet, mix well, incubate 2 min at RT.		
<input type="checkbox"/>	Add 30 µl <b>PS</b> , mix well, incubate 5 min at RT.		
<input type="checkbox"/>	Place on magnet for 2 - 5 min, remove and discard supernatant.		
<input type="checkbox"/>	Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.		
<input type="checkbox"/>	Air dry beads for 5 - 10 min. <b>ATTENTION:</b> Do not let the beads dry too long!		
<input type="checkbox"/>	Add 20 µl <b>EB</b> , remove from magnet, mix well, incubate 2 min at RT.		
<input type="checkbox"/>	Place on magnet for 2 - 5 min, transfer 15 - 17 µl of the supernatant into a fresh PCR plate. 🛑 Safe stopping point.		



## 7. Appendix A: General RNA Requirements

### Potential Contaminants

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

### Genomic DNA Contamination

Depending on the RNA extraction protocol used, samples may also contain significant amounts of genomic DNA (gDNA), which is indistinguishable from RNA on a spectrophotometer. Furthermore, as many of the dyes used in RNA microfluidics assays stain single-stranded nucleic acids more intensively than double-stranded, low to moderate amounts of gDNA may not be readily visible with an RNA-specific microfluidics assay. We highly recommend examining all RNA samples on a denaturing agarose gel or using a fluorometric assay with DNA- and RNA-specific dyes to check samples for DNA contamination. On an agarose gel gDNA can appear as either a dark mass, which remains in the slot if relatively intact, or as a high molecular weight smear if it has been sheared during extraction. QuantSeq libraries generated from samples containing gDNA may have an increased number of intergenic reads or lower strandedness.

The best way to avoid gDNA contamination is to use an RNA extraction protocol that does not co-isolate gDNA (e.g., Lexogen's SPLIT RNA Extraction Kit, Cat. No. 008.48). However, DNA can be removed from irreplaceable samples by acidic phenol extraction or DNase I digestion. While DNase I treatment may be advisable for FFPE RNA, in general we do not recommend DNase treatment, as the extended incubation with divalent cations can lead to RNA hydrolysis and decrease RNA integrity. If samples must be DNase treated, heat inactivation should be avoided, and the enzyme should be deactivated by other means such as phenol / chloroform extraction or silica column purification.

### RNA Integrity

The integrity of an RNA sample can be assessed with a variety of methods. We recommend the use of a microfluidics assay such as the RNA6000 series for the 2100 Bioanalyzer (Agilent Technologies, Inc.), although RNA quality can also be assessed with denaturing agarose gel electrophoresis if such a device is not available. Most microfluidics platforms will carry out an automated peak analysis and generate a quality score (RIN or RQN). As QuantSeq specifically targets the 3' end of

transcripts even RNAs with a lower RIN are suitable as input material.

## Mitochondrial Ribosomal RNA

Mitochondrial ribosomal RNAs (mt-rRNAs) are polyadenylated and hence will also be reverse transcribed and converted into a cDNA library. mt-rRNAs can make up 1 - 2 % of the reads when using a 3' mRNA-Seq protocol, such as QuantSeq, as only one fragment will be generated for each transcript. Optional an rRNA depletion method, which also removes mt-rRNAs, such as Lexogen's RiboCop rRNA Depletion Kit (Cat. No. 037), can be used before starting the QuantSeq library preparation if it is essential to remove mt-rRNA transcripts.

## RNA Storage

If immediate RNA extraction is not possible, tissue samples can be either flash-frozen with liquid nitrogen or submerged in RNAlater (Life Technologies, Inc.) and stored at -80 °C. After extraction, RNA can be stored at -20 °C or -80 °C in 10 mM Tris pH 7.0. Addition of RNasin or an equivalent RNase inhibitor is recommended. Avoid frequent freeze-thaw cycles as RNA might be sheared.

## SIRVs Spike-In RNA Variant Control Mixes

Lexogen offers a set of artificial spike in transcripts called SIRVs (Spike-In RNA Variants), to serve as a control and anchor set for the comparison of RNA-Seq experiments. The SIRVs consist of 69 artificial RNA transcripts with no identity to any known genomic sequences, hence they can be spiked into any RNA. SIRVs are available in three sets, SIRV-Set 1 (Cat. No. 025) contains the Isoform Mixes E0, E1, and E2 of the isoform module, SIRV-Set 2 (Cat. No. 050) provides the Isoform Mix E0 only, whereas SIRV-Set 3 (Cat. No. 051) has the SIRV Isoform Mix E0 in a mixture with the ERCC RNA Spike-in controls (Ambion Inc., see below). The SIRVs are polyadenylated mRNAs and therefore are efficiently captured during QuantSeq 3' mRNA-Seq 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 (Ambion Inc.). For QuantSeq we recommend using SIRV-Set 3 (Cat. No. 051), which contains ERCCs together with the SIRV isoform controls. ERCCs have a known strand orientation and no antisense transcripts, so the calculation of strandedness based on ERCC sequences is more accurate than calculations based on reads aligned to the genome. The input-output correlation can be computed by comparing the given concentrations of the ERCC RNA Spike-in transcripts with their expression value in the sequenced library. Any potential overcycling of the libraries can be detected. Transcripts may have different and not yet annotated 3' ends, which might be mistaken for internal priming events of the oligo(dT) primer, when in fact those are true 3' ends. As ERCC transcripts only have one defined 3' end, this provides the only true measure to determine internal priming.

## 8. Appendix B: RNA Input and PCR Cycles

The amount of total RNA required as input for QuantSeq depends on the poly(A) RNA content of the sample in question and RNA quality. In general, high quality mRNA-Seq data relies on high quality input RNA. However, as QuantSeq is a 3' mRNA-Seq protocol both high and low quality RNA can be used as input.

### Input Guidelines

- The minimum recommended input amount of high-quality total RNA is 100 pg for QuantSeq FWD HT Library Prep.
- The maximum input is 2 µg.
- As a starting point, we recommend performing the protocol initially with 500 ng total RNA. RNA inputs ≥200 ng are recommended to detect low abundant transcripts efficiently.
- Lower RNA inputs (≤10 ng), and low quality RNA samples require protocol modifications, including adjusting the number of PCR cycles for the endpoint PCR (see Appendix C, p.26 and Appendix D, p.27).
- QuantSeq is also highly suitable for FFPE samples. For further details on FFPE samples refer to Appendix D (p.27).
- We recommend performing a qPCR assay to determine the optimal number of PCR cycles for library amplification. In particular, whenever different sample types (e.g., species, tissue), samples with varying RNA quality, and low input amounts (≤10 ng) are used.

As a guideline, PCR cycle numbers and key parameters of libraries synthesized with Lexogen's QuantSeq kit using different input RNA amounts of high-quality Universal Human Reference RNA (UHRR) are shown in the table below. **These reference values are provided as a guideline only.** For other sample types, yields may vary and PCR cycle numbers may need to be adjusted.

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

\*All libraries were prepared with dual indexing. Linker sequences are 132 bp including 6 nt long i5 and i7 indices, respectively. For single Indexing (i7 only) adapter sequences are 122 bp. \*\* Not recommended for QuantSeq REV (Cat. No 016).

## Adjusting PCR Cycle Numbers for Sample Type

Depending on the mRNA content of the RNA sample, which can vary between tissues for the same species, and also between species, the number of PCR cycles used for library amplification should be adjusted. Lexogen provide a PCR Add-on Kit (Cat. No. 020) and qPCR assay protocol (see Appendix E, p.28) that can be used to calculate the optimal number of PCR cycles for the specific RNA input.

QuantSeq was tested extensively with 500 ng of high quality total RNA from various cell cultures, mouse and plant tissues, yeast, and fungi, against Universal Human Reference RNA (UHRR) and Human Brain Reference RNA (HBRR) (see table below). Between 11 - 14 PCR cycles were needed for library amplification, depending on the mRNA content of the RNA in question.

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

Mm: *Mus musculus*; Sc: *Saccharomyces cerevisiae*

**These reference values are provided as a guideline only.** Libraries prepared from other RNA sample types, input amounts, or RNA with reduced quality may require different PCR cycle numbers. **We strongly recommend performing a qPCR assay to determine the optimal number of PCR cycles** for different RNA sample types (see Appendix E, p.28).

Optimizing the number of PCR cycles for the endpoint PCR will also help to prevent under- or overcycling the libraries. Undercycling may result in insufficient amounts of library for sequencing. It is essential to avoid overcycling, which is indicated by a second high molecular weight peak between 1,000 - 9,000 bp in a Bioanalyzer trace. Overcycling generates amplification artefacts and will introduce bias into the sequencing data.

## 9. Appendix C: Low Input RNA

When working with low input RNA ( $\leq 10$  ng), low quality or degraded RNA, or RNA isolated from Formalin-Fixed Paraffin Embedded (FFPE) samples, only minor protocol modifications are recommended for optimal library prep consistency and yield. Different protocol modifications apply when using input amounts  $\leq 10$  ng, or  $\leq 1$  ng, or when using FFPE RNA (see also Appendix D, p.27). These are outlined in the table below, alongside the standard protocol steps.

Protocol Step	Standard Input (> 10 ng)	Low Input ( $\leq 10$ ng) FFPE / Degraded RNA	Low Input ( $\leq 1$ ng)
Step 2	Incubate for 3 minutes at 85 °C, then cool to 42 °C. Hold samples at 42 °C on the thermocycler.	<b>Skip!</b> Prepare pre-warmed <b>FS1 / FS2 / E1</b> mastermix! Place RNA samples at room temperature.	
Step 3	Prepare <b>FS2 / E1</b> mastermix – pre-warm for 2 - 3 minutes at 42 °C.	Prepare <b>FS1 / FS2 / E1</b> mastermix – pre-warm for 2 - 3 minutes at 42 °C.	
Step 4	Add pre-warmed mastermix to <b>RNA / FS1</b> samples at 42 °C. Incubate for 15 minutes at 42 °C.	Add pre-warmed mastermix to RNA samples at room temperature. <b>OPTIONAL:</b> Increase incubation time to 1 hour at 42 °C.	
Step 6	Incubate for 10 minutes at 95 °C.	Incubate for 10 minutes at 95 °C.	Reduce to 5 minutes at 95 °C.
Step 16	Add 56 µl of Purification Solution ( <b>PS</b> ).	Reduce volume of Purification Solution ( <b>PS</b> ) to 48 µl.	
Step 24	The qPCR assay is strongly recommended when processing samples with: <ul style="list-style-type: none"> <li>• Variable input amounts</li> <li>• Variable RNA quality</li> <li>• Different or new sample types (e.g., species, tissue, cell type)</li> </ul>	The qPCR assay is strongly recommended for all low input, FFPE / degraded RNA library preps, to prevent over- or undercycling of the libraries.	
Step 29	<b>Single-indexed libraries:</b> Add 30 µl of Purification Beads ( <b>PB</b> ).	<b>Single-indexed libraries:</b> Reduce volume of Purification Beads ( <b>PB</b> ) to 27 µl.	
Step 30	<b>Dual-indexed libraries:</b> Add 35 µl of Purification Beads ( <b>PB</b> ).	<b>Dual-indexed libraries:</b> Reduce volume of Purification Beads ( <b>PB</b> ) to 31.5 µl.	

## 10. Appendix D: Low Quality RNA - FFPE

RNA isolated from Formalin-Fixed Paraffin Embedded (FFPE) samples is often heavily degraded. As QuantSeq is a 3' mRNA-Seq protocol it is highly suitable for FFPE RNA.

For FFPE samples only minor protocol adjustments are required (see also Appendix C, p.26), specifically:

- Skipping step 2.
- Reducing the addition of **PS** in step 16 to 48 µl.
- Using only 27 µl **PB** in step 29 for single indexing PCR, and 31.5 µl in step 30 for dual indexing PCR, respectively (see FAQs at [www.lexogen.com](http://www.lexogen.com)).

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

- Extending the reverse transcription time in step 4 to 1 hour (≤10 ng).
- Reducing the RNA removal time in step 6 to 5 minutes at 95 °C (≤1 ng).

As the RNA amount is often a limiting factor with FFPE samples, QuantSeq was tested with 500 pg - 50 ng FFPE or degraded RNA input. The table below comprises some results obtained from using different amounts of mouse (Mm) brain FFPE RNA input with a RIN of 1.8 (DV<sub>200</sub> of 51 %). The DV<sub>200</sub> is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV<sub>200</sub>, the more degraded the RNA is. **ATTENTION:** FFPE RNA samples are highly variable. Samples with lower mRNA content, or lower DV<sub>200</sub> values may require more PCR cycles.

**We strongly recommend performing a qPCR assay** (using Lexogen's PCR Add-on Kit for Illumina (Cat. No. 020, Appendix E, p.28) to determine the optimal cycle number for library amplification.

Input RNA (Mm brain FFPE RNA)	Step 6: RNA Removal 95°C	Step 16: PS Addition	Library*			Insert				Library Yield		PCR Cycles
			Start [bp]	End [bp]	Mean Size*	Mean Size	≥ 50 nt	≥ 100 nt	≥ 200 nt	ng/µl	nM	
50 ng	10 min	48 µl	132	600	264	132	96 %	52 %	5 %	2.3	14.2	15
10 ng	10 min	48 µl	132	600	265	133	95 %	56 %	5 %	2.1	13.1	18
500 pg	5 min	48 µl	132	600	266	134	85 %	53 %	4 %	1.2	7.5	22

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

**ATTENTION:** FFPE RNA is degraded RNA, hence the insert sizes are smaller than for non-degraded RNA samples. Keep this in mind when choosing your sequencing length.

If you see that your FFPE RNA generates a lot of linker-linker products despite the above-mentioned protocol changes, re-purification of the lane mix with 0.9x **PB** (e.g., 50 µl lane mix plus 45 µl **PB**, incubating 5 minutes at room temperature, and following the protocol from step 30 (for single-indexed libraries) onwards may be necessary.

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

# 11. Appendix E: qPCR

The mRNA content and quality of total RNA affects the number of PCR cycles needed for the final library amplification step. We strongly recommend taking advantage of the qPCR assay. Over- or undercycling may bias your sequencing results (transcript abundance estimation and library quantification) and can be avoided by optimising the PCR cycle number.

## qPCR to Determine the Optimal Cycle Number for Endpoint PCR

The PCR Add-on Kit for Illumina (Cat. No. 020.96) provides additional PCR Mix (**PCR** ●), Enzyme Mix (**E** ●), and the P7 Primer (**7000** ●) needed for the qPCR assay. In addition, SYBR Green I nucleic acid dye (Sigma Aldrich, S9430, user-provided) is also required for quantification.

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

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

---

1 Dilute the double-stranded library from step 24 to 19 µl by adding 2 µl Elution Buffer (**EB**) or molecular biology-grade water (H<sub>2</sub>O).

---

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 the amplification.

---

3 For each reaction combine: 1.7 µl of the diluted cDNA library, 7 µl of PCR Mix (**PCR** ●), 5 µl of P7 Primer (**7000** ●), 1 µl of Enzyme Mix (**E** ●), and 1.2 µl of 2.5x SYBR Green I nucleic acid dye. Make the total reaction volume up to 30 µl by adding 14.1 µl of Elution Buffer (**EB**) or molecular biology-grade water (H<sub>2</sub>O). **ATTENTION:** Include a no template control!

---

4 Perform 40 - 50 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, 45 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, hold at 10 °C **REMARKS:** 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.

---

**REMARK:** The qPCR assay can be used for dual or single indexed libraries.

500 ng total RNA input was used for generating libraries. Using 1.7  $\mu$ l of cDNA for a qPCR, the cycle number corresponding to 50 % of the maximum fluorescence was 15 cycles. The remaining 17  $\mu$ l of the template should therefore be amplified with 12 cycles (15-3 cycles = 12 cycles).



## 12. Appendix F: Library Reamplification

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

For reamplification of dual-indexed libraries the Reamplification Add-on Kit for Illumina (Cat. No. 080.96) is available on request. Please contact Lexogen at [info@lexogen.com](mailto:info@lexogen.com).



## 13. Appendix G: Library Quality Control

Quality control of finished QuantSeq libraries is highly recommended and can be carried out with various methods depending on available equipment. A thorough quality control procedure should include the analysis of both the concentration, shape, and the size distribution of libraries.

### Quality Control Methods

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

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

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

### Typical Results

QuantSeq libraries are intended for a high degree of multiplexing, and hence libraries do not need to be extensively amplified. 12 cycles of PCR amplification are usually enough when using 500 ng total RNA input. This will prevent overcycling and distorted expression values while at the same time providing enough material for quantification and subsequent cluster generation.

Typical concentrations are between 12.7 - 9.8 nM (2.4 - 1.8 ng/μl) for 50 ng (15 cycles) and 500 ng (12 cycles) input RNA, respectively, with most inserts being between 110 - 200 nt in length (see also Appendix C, p.26).

# Overcycling

A second peak in high molecular weight regions (between 1,000 - 9,000 bp) is an indication of overcycling. This could occur if cycle numbers are increased too much to compensate for lower input material. Prevent overcycling by using the qPCR assay as described in Appendix E, p.28. Lexogen's PCR Add-on Kit for Illumina (Cat. No. 020) offers the option to reamplify undercycled single indexed libraries. For more details please refer to Appendix E, p.28 as well as the PCR Add-on Kit Instruction Manual. For reamplification of dual-indexed libraries contact Lexogen at [info@lexogen.com](mailto:info@lexogen.com).

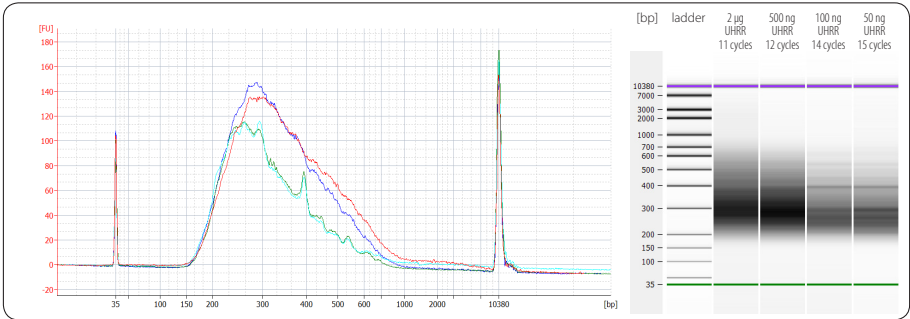


Figure 4. Bioanalyzer trace of QuantSeq FWD HT libraries synthesized from 2 μg (red trace), 500 ng (dark blue trace), 100 ng (green trace) and 50 ng (light blue trace) total RNA input (Universal Human Reference RNA, UHRR) amplified with 11 cycles, 12 cycles, 14 cycles, and 15 cycles, respectively. Dual indexing was used for all libraries.

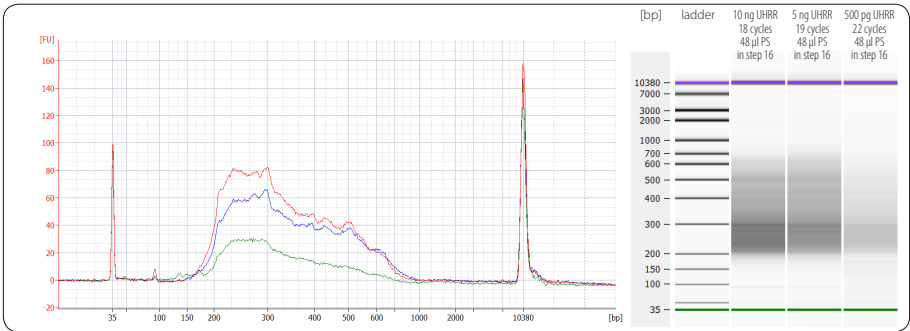


Figure 5. Bioanalyzer trace of QuantSeq FWD HT libraries synthesized from 10 ng (red trace), 5 ng (blue trace), and 500 pg (green trace) total RNA input (Universal Human Reference RNA, UHRR) amplified with 17 cycles, 18 cycles, and 22 cycles, respectively. Step 2 was skipped for all samples and for 500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

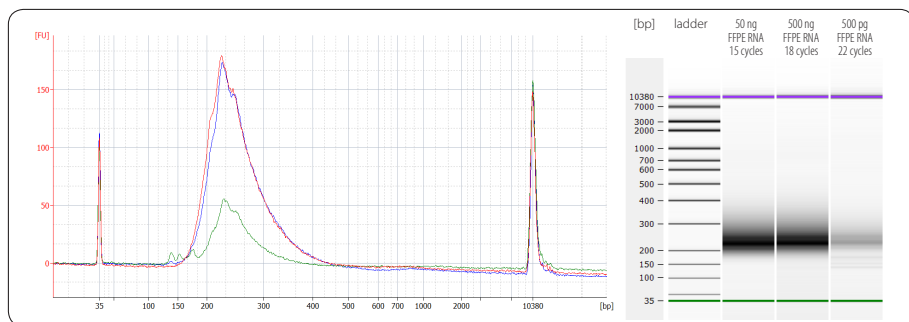


Figure 6. Bioanalyzer traces of QuantSeq FWD HT libraries synthesized from 50 ng (red trace), 10 ng (dark blue trace), and 500 pg (green trace) mouse (Mm) brain FFPE RNA (RIN 1.8, DV<sub>200</sub> 51 %). All libraries were prepared with the recommendations for FFPE RNA input. 500 pg FFPE RNA libraries already contain some artifacts below 150 bp which should be removed before sequencing, e.g., by repurifying the lane mix (see Appendix D, p.27).

# 14. Appendix H: Modulating Insert Sizes

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

In short: **FS1** and **FS2** from the basic QuantSeq FWD kit are exchanged with **FS1x**, **FS2x**, and **dT** from the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026). Due to buffer differences and protocol changes, longer inserts can be generated.

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

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

\*All libraries were prepared from 500 ng Universal Human Reference RNA (UHRR) with dual indexing. Linker sequences are 132 bp including 6 nt long i5 and i7 indices, respectively. FS1x, FS2x, and oligo dT (dT) are components from the QuantSeq-Flex First Strand Synthesis Module (Cat. No. 026). FS1 and FS2 are from the QuantSeq FWD HT basic kit.

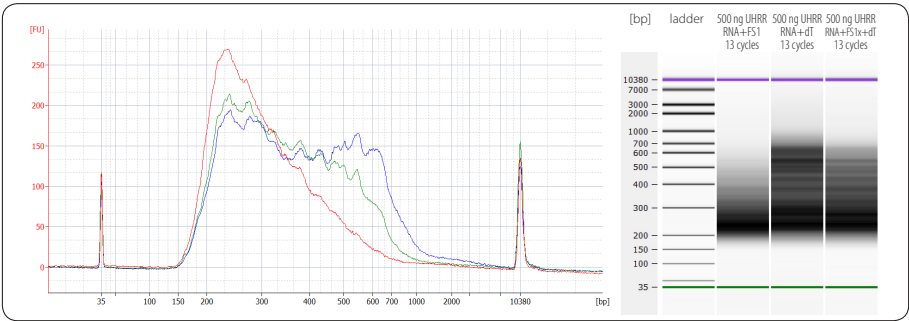


Figure 7. Bioanalyzer traces of QuantSeq FWD HT libraries with library size modulation. The shortest insert sizes are generated with the basic QuantSeq kit components, i.e., denaturing of RNA in FS1 (red trace, standard protocol). With the QuantSeq-Flex First Strand Synthesis Module and denaturing RNA and oligo(dT) (dT) only, before adding FS1x, FS2x, and E1 the longest average insert sizes are generated (dark blue trace). With the QuantSeq-Flex First Strand Synthesis Module and denaturing RNA, FS1x, and dT before adding FS2x and E1 the average insert size can be increased (green trace). Input RNA was 500 ng Universal Human Reference RNA (UHRR) for all libraries. Dual indexing with 13 cycles of final library amplification was used for all libraries.

# 15. Appendix I: Globin Block Modules

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

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

The Globin Blocker oligos anneal to the 3' ends of globin first strand cDNA downstream of the random primers and thereby, prevent the generation of amplifiable library fragments from globin mRNAs during second strand synthesis (Fig. 8).

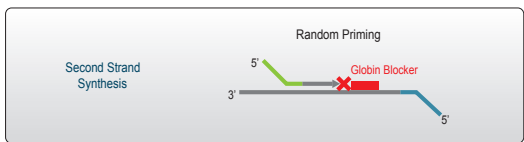


Figure 8. Globin Block Module for QuantSeq workflow.

## Kit Components and Storage Conditions

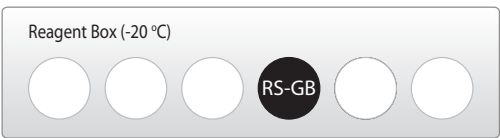


Figure 9. Location of kit components.

Kit Component	Tube Label	Volume* 96 rxn	Storage
Removal Solution-Globin Block, <i>Homo sapiens</i> , 96 rxn (Cat. No. 070.96)	RS-GBHs ●	528 µl	-20 °C
Removal Solution-Globin Block, <i>Sus scrofa</i> , 96 rxn (Cat. No. 071.96)	RS-GBSs ●	528 µl	-20 °C

\*including 10 % surplus

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

# Short Protocol: RNA Removal Globin Block

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

Follow steps 1 to 4 of the detailed protocol (p.11-12).

- 5 Add 5 µl of RS-Globin Block (RS-GB ●), directly to the first strand cDNA synthesis reaction. Mix well and reseal the plate using a fresh foil. **REMARK:** Use a pipette set to 15 µl for efficient mixing.
- 6 Incubate 10 minutes at 95 °C, then cool down to 25 °C. Spin down the plate at room temperature and carefully remove the sealing foil. **ATTENTION:** Reduce this step for RNA inputs ≤1 ng total RNA to 5 minutes at 95 °C (see Appendix C, p.26).

➡ Proceed with steps 7 to 43 of the detailed protocol (p.13-19).

## RNA Input and Library Amplification

The minimum recommended input for QuantSeq using Globin Block Modules is 50 ng of total RNA from whole blood, or leukocyte-enriched blood (i.e. after red blood cell lysis).

The table below shows an example of PCR cycle numbers used for different input amounts of human and pig total RNA from blood. These cycle numbers should be interpreted as a guideline only. **We strongly recommend performing a qPCR assay to determine the optimal number of cycles for library amplification** (see Appendix E, p.28).

Species	Whole Blood or Leuko- cyte-Enriched Blood	Input Amount	PCR Cycles	
			Undepleted	+ RS-Globin Block
Human	Whole Blood	250 ng	13	14
		50 ng	15	16
	Leukocyte-Enriched Blood	50 ng	16	17
		50 ng*	15	16
Pig	Whole Blood	100 ng	16	17

\* Indicates RNA isolated using PAXgene® Blood System (Tubes and Kit, Qiagen), which includes 24-hour red blood cell lysis. Pig blood was collected in Tempus blood RNA tubes and extracted using the Preserved Blood RNA Purification Kit I + DNase I Kit (Norgen Biotek). All other RNA was extracted using the SPLIT RNA Extraction Kit (Lexogen, Cat. No. 008). All libraries were prepared with single indexing. Linker sequences are 122 bp including 6 nt long i7 indices.

## Typical Results

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

# 16. Appendix J: Unique Molecular Identifiers

Unique Molecular Identifiers (UMIs) can be included in QuantSeq FWD libraries to enable the detection and removal of PCR duplicates. The UMI Second Strand Synthesis Module for QuantSeq FWD (Illumina, Read 1) (Cat. No. 081) contains the UMI Second Strand Synthesis Mix (**USS** ●), which contains UMI-tagged random primers. The **USS** ● simply replaces the Second Strand Synthesis Mix 1 (**SS1** ●) from the standard QuantSeq FWD Kit. No other protocol changes are required. The UMIs are added between the partial P5 adapter and the random priming sequence, during second strand synthesis (Fig. 10).



Figure 10. UMIs are added during the second strand synthesis step of the QuantSeq workflow.

## Kit Components and Storage Conditions



Figure 11. Location of kit component.

Kit Component	Tube Label	Volume* 96 rxn	Storage
<b>UMI Second Strand Synthesis Mix</b> (Cat. No. 081.96)	<b>USS</b> ●	1,056 µl	-20 °C

\*including 10 % surplus

### ATTENTION:

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

# Short Protocol - Second Strand Synthesis

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

Follow steps 1 - 6 of the detailed protocol (p.11-13).

7 Add 10 µl of UMI Second Strand Synthesis Mix (USS ●) to the reaction. Mix well by pipetting, and seal the plate. **REMARK:** Use a pipette set to 30 µl for efficient mixing.

8 Incubate the plate for 1 minute at 98 °C in a thermocycler, and slowly cool down to 25 °C by setting the ramp speed to 10 % (0.5 °C/second). Incubate the reaction for 30 minutes at 25 °C. Quickly spin down the plate at room temperature before removing the sealing foil.

➡ Proceed to step 9 of the detailed protocol (p.13).

## Sequencing

A minimum length of 75 bp (i.e., SR75 or longer) is recommended for sequencing QuantSeq FWD libraries that include UMIs. The 6 nt UMI is read-out at the beginning of Read 1, upstream of the random priming sequence (Fig. 11). QuantSeq libraries containing UMIs are compatible with dual indexing using the i5 Dual Indexing Add-on Kits for QuantSeq/SENSE (5001-5096) (Cat. No. 047). No custom sequencing primers are required.

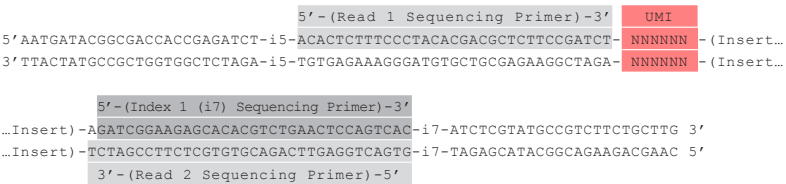


Figure 12. QuantSeq FWD libraries with UMIs.

## Data Analysis

Sequencing data containing UMIs can be analyzed using the QuantSeq Data Analysis pipeline available on the Bluebee® Genomics Platform. An additional command-line analysis tool (**collapse\_UMI\_bam**) is also available for de-duplication of sequencing read counts using UMIs. To obtain a copy of the source code, or for further information on UMI data analysis methods, please contact [info@lexogen.com](mailto:info@lexogen.com).



# 17. Appendix K: Multiplexing

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

## i7 Indices

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

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

i7 index sequences are available for download at [www.lexogen.com](http://www.lexogen.com).

In general, we recommend processing a minimum of 8 samples, using a complete set of eight i7 indices for multiplexing (e.g., **7001-7008**). However, if fewer indices are required care should be taken to always use indices which give a well-balanced signal in both lasers (red and green channels) for each nucleotide position. All columns (1 - 12) and rows (A - H) fulfill these criteria when individual libraries are mixed in an equimolar ratio. Use the online Index Balance Checker tool available at <https://www.lexogen.com/support-tools/index-balance-checker/>, to select the ideal combination of indices for optimal color and nucleotide balance.

**REMARK:** If an 8 nt i7 index (Index 1) needs to be entered into an Illumina sample sheet, e.g., if QuantSeq libraries are multiplexed with 8 nt indexed libraries from other NGS-kit suppliers, add two nucleotides from the Illumina adapter sequence to the 3' end of the index.

**EXAMPLE:** **7001** would become CAGCGT**AT**, **7002** would become GATCAC**AT** and so on. These additional nucleotides are identical for all indices as they are derived from the Illumina adapter.

# i5 Indices

The QuantSeq 3' mRNA-Seq Library Prep Kit FWD HT (Cat. No. 015.384) includes 4 perfectly balanced i5 Indices (**5001-5004** ●). Dual-indexed libraries prepared with these i5 indices are suitable for sequencing on all Illumina instruments listed below. Each nucleotide (A, C, G, and T) is present at each position of the 6 nt long index, resulting in perfect nucleotide and color balance.

Alternatively, the i5 Unique Dual Indexing Add-on Kit for QuantSeq/SENSE (5001-5096, Cat. No. 047.96) provides the 96-well i5 Index Plate that contains 96 unique i5 indices **5001-5096**.

The i5 index is sequenced during the Index 2 Read. Depending on the instrument workflow and flow cell type (paired-end, PE; single-read, SR), i5 indices are sequenced in either forward or reverse complement. This affects how the i5 index sequences need to be entered into the sample sheets. These details are summarized in the following table.

Index 2 (i5) Oligo	i5 Sequences for Sample Sheet Entry	
	Forward Orientation (i5) MiSeq®, HiSeq® 2000/2500 (SR/PE), HiSeq® 3000/4000 (SR), NovaSeq™	Reverse Complement Orientation (i5rc) MiniSeq™, NextSeq™, HiSeq® 3000/4000 (PE)
<b>5001</b> ●	CGCCAT (AC)	ATGGCG (GT)
<b>5002</b> ●	ATTTTA (AC)	TAAAT (GT)
<b>5003</b> ●	GCAACG (AC)	CGTTGC (GT)
<b>5004</b> ●	TAGGGC (AC)	GCCCTA (GT)

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

**REMARK:** Some Illumina sample sheets may require 8 nt to be entered for Index 2. In this case, add two nucleotides from the Illumina adapter sequence to the 3' end of the index e.g., **5001** becomes CGCCAT**AC** on a MiSeq® and ATGGCG**GT** on a NextSeq™, respectively.

All i5 index sequences are available for download at [www.lexogen.com](http://www.lexogen.com).

## Dual Indexing Strategies

Each of the 5 i5 indices can be combined with any of the i7 indices provided within the i7 Index Plate (**7001-7096**) of the QuantSeq 3' mRNA-Seq Kit FWD HT for Illumina (Cat. No. 015.384). By combining the four different i5 indices with the 96 different i7 indices, 384 different index combinations can be achieved.

Using the full set of 96 i5 indices provided in the i5 Unique Dual Indexing Add-on Kit for QuantSeq/SENSE (5001-5096, Cat. No. 047.96), together with the 96 i7 indices provided in Lexogen's i7 Index Plate (**7001-7096**), a maximum of 9,216 different i5 / i7 index combinations can be achieved. Alternatively, up to 96 uniquely dual-indexed libraries can be multiplexed for sequencing, without repeating the use of any of the i5 or i7 indices.

## Index Balance

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

The index balance should be checked for both the i5 and i7 index reads separately when designing the multiplexing strategy for dual-indexed libraries. To determine the optimal combinations of indices (i5 and i7) for the specific number of libraries to be sequenced, we recommend using Lexogen's online Index Balance Checker tool, available at [www.lexogen.com/support-tools/index-balance-checker/](http://www.lexogen.com/support-tools/index-balance-checker/).

In general, using the i5 indices in numerical order as the number of libraries to multiplex increases, or column-wise for increasing multiples of 8 samples, will result in optimal nucleotide balance for Index 2 Read (i5). For smaller numbers of samples we can also suggest the following:

- **Four libraries:** Use indices **5001 - 5004**, as these contain perfect nucleotide balance at each position of the index read.
- **Eight libraries:** Use column 1 of the i5 Index Plate (indices **5001 - 5008**).

For suggestions and guidelines regarding i7 index selection for multiplexing please also refer to the QuantSeq 3' mRNA-Seq (015UG009), or QuantSeq-Flex (015UG058) User Guides.

**NOTE:** Individual libraries within a lane or run should always be pooled at an equimolar ratio to preserve perfect nucleotide balance at each position of the index read. If non-equimolar library pooling is required, the relative molarity of each library can be adjusted using the Index Balance Checker tool, when selecting indices to use for your libraries.

# 18. Appendix L: Sequencing\*

## General

The amount of library loaded onto the flow cell will greatly influence the number of clusters generated. Each sequencing facility has slightly different preferences of how much to load. Machine-specific loading instructions can be found at [www.lexogen.com](http://www.lexogen.com) under QuantSeq Frequently Asked Questions (FAQ 1.24 (FWD)).

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

## Single Indexing (i7 only) - All Illumina Platforms

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

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

```
5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert...
3'TTACTATGCCGCTGTTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert...

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

**Read 1:** Multiplexing Read 1 Sequencing Primer (not supplied):

5'ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

**Index 1 Read (i7):** Multiplexing Index 1 (i7) Sequencing Primer (not supplied):

5'GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

## Dual Indexing

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. In general there are four different workflows used for Index 2 Read (i5) read-out on Illumina instruments, which are summarized in the table below.

Flow Cell Type	Workflow <sup>1</sup>	Instrument(s)	i5 Index Read Primer	i5 Index Read Orientation	No. Chemistry-Only Cycles <sup>2</sup>
SR	1	HiSeq® 2000/2500	Multiplexing Index 2 (i5) Sequencing Primer (HP9)	Forward (i5)	0
	2	HiSeq® 3000/4000	Grafted P5 Oligo		7
	3	HiSeq® 2000/2500 MiSeq® NovaSeq™ 6000			
PE	4	MiniSeq™ NextSeq® 500/550	Multiplexing Index 2 (i5) Sequencing Primer (HP14)	Reverse complement (i5rc)	0
		HiSeq® 3000/4000	Multiplexing Index 2 (i5) Sequencing Primer (dual-indexing primer mix)		

<sup>1</sup>Workflows 3 and 4 correspond to Illumina's Dual-Indexed Workflows A and B on paired-end flow cells, respectively.

<sup>2</sup>Additional chemistry-only (no-imaging) cycles are performed before the i5 index is read-out.

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

The order of sequencing for Workflow 4 is: Read 1, index read preparation, Index 1 Read (i7), Read 2 Resynthesis, and Index 2 Read (i5), plus Read 2 for paired-end runs.

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

## Dual Indexing Sequencing Workflows

The following schemes depict the library adapters and sequencing primer binding sites for dual-indexed QuantSeq FWD HT libraries. The sequences of the relevant Index 2 Read (i5) Sequencing Primers are also provided.

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

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

The Index 2 (i5) Sequencing Primer (included in HP9) is required for Index 2. A minimum of six cycles with imaging are required for the i5 read.

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

### QuantSeq FWD, QuantSeq-Flex, SENSE mRNA, and SENSE Total libraries:

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

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

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

The Grafted P5 Oligo is used to initiate index 2 (i5) read-out. Seven additional chemistry-only cycles (no imaging) plus a minimum of six cycles (with imaging) are required to read the i5 index.

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

```
5'-(Grafted P5 Oligo)-3'      5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert...
3'TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert...

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

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

The Grafted P5 Oligo is used to initiate index 2 (i5) read-out. Seven additional chemistry-only cycles (no imaging) plus a minimum of six cycles (with imaging) are required to read the i5 index.

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

```
5'-(Grafted P5 Oligo)-3'      5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert...
3'TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert...

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

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

All instruments use a Multiplexing Index 2 (i5) Sequencing Primer, which is included in the “Dual-Indexing Primer Mix” for MiniSeq™ and NextSeq®, and in HP14 for HiSeq® 3000 / 4000. A minimum of six cycles (with imaging) are required to read the i5 index.

**ATTENTION:** Index 2 (i5) is read-out after the Read 2 Resynthesis step, hence a reverse complement of the Index 2 (i5) primer sequence is produced (see also Appendix A, p.22).

**Index 2 Read (i5):** Multiplexing Index 2 (i5) Sequencing Primer (not supplied):

5' AGATCGGAAGAGCGTCGTAGGGAAAGAGTGT 3'

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

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

# 19. Appendix M: Data Analysis

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

QuantSeq is available in two read orientations: QuantSeq FWD (Cat. No. 015) contains the Read 1 linker sequence in the 5' part of the second strand synthesis primer, hence NGS reads are generated towards the poly(A) tail. To pinpoint the exact 3' end, longer read lengths may be required. Read 1 directly reflects the mRNA sequence.

I

For more detailed information please refer to [www.lexogen.com/quantseq-data-analysis](http://www.lexogen.com/quantseq-data-analysis).

## Processing Raw Reads

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

## Demultiplexing

Demultiplexing can be carried out by the standard Illumina pipeline. i5 and i7 index sequences are available for download at [www.lexogen.com](http://www.lexogen.com).

## Trimming

As second strand synthesis is based on random priming, there may be a higher proportion of errors at the first nucleotides of the insert due to non-specific hybridization of the random primer to the cDNA template. These mismatches can lead to a lower percentage of mappable reads when using a stringent aligner such as TopHat2, in which case it may be beneficial to trim these nucleotides. For QuantSeq FWD (Cat. No. 015) the first 12 nucleotides of Read 1 need to be removed. Alternatively, a less stringent aligner (e.g., STAR Aligner) could be used with the number of allowed mismatches being set to 14, which removes the need to trim the first 12 nucleotides. While trimming the first nucleotides can decrease the number of reads of suitable length, the absolute number of mapping reads may increase due to the improved read quality. Reads, which are too short or have generally low quality scores should also be removed from the set.

## Alignment

At this point the filtered and trimmed reads can be aligned with a short read aligner to the reference genome. STAR aligner with mismatch allowance, or TopHat2 (after trimming nucleotides 1 - 12) can be used for mapping **QuantSeq FWD** (Cat. No. 015) data. The reads may not land in the last exon and span a junction, hence splice-aware aligners should be used.



## Annotation

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

More information about the principal data analysis method can be found under [www.lexogen.com/quantseq-data-analysis](http://www.lexogen.com/quantseq-data-analysis).

## Integrated Data Analysis Pipeline at Bluebee®

Each purchased QuantSeq kit includes a code for free data analysis including differential expression (DE) analysis using the Bluebee® Genomics Platform. Please visit [www.bluebee.com/lexogen/](http://www.bluebee.com/lexogen/) for more information and to access the data analysis pipeline. To login, enter the code received with the kit. For further inquiries, please contact [info@lexogen.com](mailto:info@lexogen.com).

For details of the technical parameters used for QuantSeq data analysis pipelines on the Bluebee platform, please see the QuantSeq 3' mRNA-Seq Integrated Data Analysis Pipeline on Bluebee Platform User Guide, available online from [www.bluebee.com/lexogen](http://www.bluebee.com/lexogen).

## 20. Appendix N: Revision History

The revision history table below shows user guide versions and kit changes made from 2016 onwards. The full revision history is available from [www.lexogen.com](http://www.lexogen.com).

Publication No./ Revision date	Change	Page
<b>015UG110V0110</b> Jun. 14, 2018	PCR Mix was renamed to Dual PCR Mix (no change to buffer composition) and kit component figure updated.	6, 17-18
	Added note to pre-warm the FS2 / E1 mastermix for 2 -3 minutes at 42 °C at step 2.	11
	Safe stopping point removed after step 4: Proceed immediately to RNA removal.	12
	Updated text for Appendices B and C and added table of protocol modifications for low input.	24-26
	Modified format and text for Appendix E: qPCR, and F: Library Reamplification.	27-28
	Added new Appendices I: Globin Block Modules, and J: Unique Molecular Identifiers.	34-37
	Updated information on dual indexing, multiplexing, and sequencing workflows in Appendices K and L.	38-44
	Reduced Revision History table to show updates from 2016 onwards.	47
<b>015UG110V0102</b> Jul. 31, 2017	Front page and SIRVs text updated.	0, 22
	After denaturing (step 2), leave the reactions on the thermocycler at 42 °C until step 4.	11, 19-20
	Dry beads only at room temperature after ethanol washes.	13, 16, 18-20
	Added note that up-to-date recommendations can be found on the QuantSeq FAQ webpage.	26
<b>015UG110V0101</b> Mar. 8, 2017	Do not cool the FS2/E1 mastermix.	11
	Safe stopping points.	16, 18 - 20, 26
<b>015UG110V0100</b> Feb. 7, 2017	Release of QuantSeq FWD HT Cat. No. 015.384 with included (optional) i5 Dual Indexing Add-on Kit.	
	Barcode Plate (BC) was rearranged for improved balance and renamed to i7 Index Plate (7001-7096). Previous BC05: TAATCG replaced by 7025: TTTATG to avoid overlap with Illumina-specific indices.	4-6, 14-17, 19-20, 31-35
	RS1, renamed to RS. No more RS2 solution, hence one step less in the protocol.	12, 19, 20
	A new SS1 solution (now only 10 µl have to be added not 15 µl as previously).	12, 19, 20
	The total volume of the second strand synthesis reaction is now 40 µl (previously it was 50 µl).	12, 19, 20
	Post second strand synthesis only 16 µl PB have to be added (previously it was 20 µl).	13, 19, 20
	In step 16 (was previously step 17) only 56 µl PS have to be added (previously it was 72 µl).	13, 19, 20
	For dual indexing the PCR buffer from the basic kit (yellow cap) has to be exchanged with the PCR buffer (purple cap) included in the i5 Dual Indexing Add-on Kit.	16, 20
	Dual indexing PCR has a final volume of 35 µl (i7 single indexing PCR has a final volume of 30 µl).	17, 20
	Post dual indexing PCR 35 µl PB added (or 31.5 µl PB for low input, low quality FFPE RNA samples).	17, 20
	Table with reference values for 500 ng input RNA from different species.	25
	Barcode 00 (BC00) in PCR Add-on Kit renamed to P7 Primer 7000.	27
	qPCR endpoint determination using only 1.7 µl template and set to 50 % FU (previously 33 %). Subtract 3 cycles from determined endpoint (EP) when using 10x as much template (17 µl in EP, 1.7 µl in qPCR).	27
	Evaluation tool to check the color balance of index subsets.	32
	015.384 includes i5 Dual Indexing Add-on Kit (four i5 indices). Information on i5 and dual indexing.	33 - 36
Mar. 24, 2014	Initial Release.	

The background of the lower half of the page features a decorative graphic of several translucent blue spheres of varying sizes, connected by thin, light blue lines, creating a network-like structure.

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

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