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AUTO QUANT™ SEQ

Sequencing that counts

3′ mRNA-Seq Library Prep Kit on the Hamilton Microlab STAR Workstations

User Guide

Catalog Numbers:
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)
019 (Dummy Automation Module for QuantSeq 3′ mRNA-Seq)
020 (PCR Add-on Kit for Illumina)
022 (Purification Module with Magnetic Beads)
025 (SIRVs Spike-in RNA Variant Control Mixes)
026 (QuantSeq-Flex First Strand Synthesis Module)
028 (QuantSeq-Flex Second Strand Synthesis Module)
033 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit with First Strand Synthesis Module)
034 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit with Second Strand Synthesis Module)
035 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit with First and Second Strand Synthesis Module)

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1. Introduction

The autoQuantSeq is an automated all-in-one library preparation protocol designed to generate up to 48 Illumina-compatible libraries from polyadenylated RNA within less than 8.5 hours. It runs on the Hamilton Microlab STAR Line liquid handlers. autoQuantSeq is the automated version of the QuantSeq protocol developed by Lexogen and uses the same reagent kit. Therefore, the following information on the QuantSeq kit features also applies to autoQuantSeq[1].

The QuantSeq protocol generates only one fragment per transcript, resulting in extremely accurate gene expression values, and the sequences obtained are close to the 3' end of the transcripts.

QuantSeq is available with two read directions: QuantSeq Forward (FWD, Cat. No. 015) contains the Read 1 linker sequence in the second strand synthesis primer, hence NGS reads are generated towards the poly(A) tail and directly correspond to the mRNA sequence. To pinpoint the exact 3' end longer reads may be required. Although paired-end sequencing is possible, we do not recommend it for Cat. No. 015. 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.

For QuantSeq Reverse (REV, Cat. No. 016) the Read 1 linker sequence is introduced by the oligodT primer. Here, a Custom Sequencing Primer (CSP, included in the kit) is required for Read 1. The sequence generated during Read 1 corresponds to the cDNA. If paired-end sequencing is desired we strongly recommend using QuantSeq REV (Cat. No. 016) and the CSP for Read 1. With QuantSeq REV and the CSP it is possible to exactly pinpoint the 3' end in Read 1.

Both QuantSeq FWD and QuantSeq REV maintain strand-specificity and allow mapping of reads to their corresponding strand on the genome, enabling the discovery and quantification of antisense transcripts and overlapping genes. Multiplexing of libraries can be carried out using up to 96 external barcodes (but only up to 48 libraries can be produced in one automated run).

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.25).

Library generation is initiated by oligodT 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, PE50, SR100, PE100).

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 F, p.33, for a schematic representation of the finished library). Library quantification can be performed with standard protocols and is further discussed in Appendix D (p.30). Libraries are compatible

[1] Throughout this User Guide, information referring to the QuantSeq kit also applies to autoQuantSeq.

with single-end or paired-end sequencing. Barcodes can be introduced during the PCR amplification step as standard external barcodes (Appendix E, p.32). Data can be analyzed with a number of standard bioinformatic pipelines. Special considerations for the analysis of QuantSeq data, such as read orientation, are presented in Appendix G (p.36).

autoQuantSeq on Hamilton STAR is typically used for preparing 48 barcoded libraries. The liquid handler program allows for processing of samples in any number from 1 to 48 preps. A set of 96 barcodes is included in the QuantSeq kit in a 96-well barcode plate, a single kit can be used for several machine runs until all the barcodes are used up.

The setup of the machine run involves preparation of simple master mixes from the kit components, aliquoting the reagents to the respective plates and tubes, and placing the plates and tubes on deck. A Microsoft Excel workbook (included in the software distribution part of the kit) contains easy-to-follow preparation guidelines and recipes for master mix preparations. The workbook automatically adjusts the recipes to the number of samples processed.

A Microsoft Excel workbook (included in the software distribution part of the kit) contains easy-to-follow preparation guidelines and recipes for master mix preparations. The workbook automatically adjusts the recipes to the number of samples processed.

Thermal treatment of the samples is done off-deck, using an external thermocycler:

The full autoQuantSeq on HamiltonSTAR protocol (48 samples) can be run in 8.5 hours in total, including about 1 hour of manual setup time.

The protocol can be installed on a Hamilton Microlab STAR or STARlet system which has been hardware-configured to run NGS library preps. The hardware and software pre-configuration is identical to that of the automated library preparation using NEBNext Ultra DNA Library Prep kit for Illumina (E7370), implemented by Hamilton Robotics GmbH. Ask Lexogen for installation details.

Running an installed protocol is a simple laboratory task which only requires standard skills and know-how covered by the basic introductory training, which is part of the liquid handler installation. Installation of a new protocol on the liquid handler is, however, a task requiring certain additional skills and experience. Ask Lexogen for installation support as well as for any user-specific kit customization, such as different barcode-to-well allocation, different kit sizing, or similar.

2. autoQuantSeq Library Prep Workflow Overview

Phase 1 – PrePCR

Run Preparation

The operator has to fill all tubes, reservoirs, and microplates with all the reagents according to the instructions in the workbook. The deck has to be prepared according to the instructions.

First Strand cDNA Synthesis – Reverse Transcription

In this step an oligodT primer containing an Illumina-compatible sequence at its 5' end is hybridized to the RNA and reverse transcription takes place.

The reagent **FS1** (5 µl) is transferred from the source tube (1.5 ml tubes are used for most reagents) to the wells with samples (5 µl) and mixed.

An RNA denaturation step follows (this can be optionally deactivated for FFPE or low quality samples, see Appendix C, p.29): The machine stops and asks the operator to seal the sample plate with a film and do thermal treatment on an external thermocycler (Intervention #1: 85 °C for 3 minutes, then cooling down to 42 °C). The thermally treated plate is to be placed on deck unsealed (after centrifugation) and the run is to be resumed.

If RNA denaturation step is deactivated (e.g., for low quality RNA – FFPE) , the machine runs without interruption.

The **FS2 / E1** reagent (10 µl, prepared manually from the First Strand cDNA Synthesis Mix 2, **FS2,** 9.5 µl, and the Enzyme Mix 1, **E1**, 0.5 µl) is transferred from the source tube to the wells with samples and mixed.

The machine stops and asks the operator to seal the sample plate with a film and do thermal treatment (Intervention #1A: 42 °C for 15 minutes). The thermally treated plate is to be placed on deck unsealed (after centrifugation) and the run is to be resumed.

RNA Removal

During this step the RNA template is degraded which is essential for efficient second strand synthesis.

The **RS1** reagent (5 µl) is transferred from the source tube to the wells with samples and mixed.

The machine stops and asks the operator to seal the sample plate with a film and do thermal treatment (Intervention #2: 95 °C for 10 minutes, then cooling down to 25°C). The thermally

treated plate is to be placed on deck unsealed (after centrifugation) and the run is to be resumed.

The **RS2** reagent (5 µl) is transferred from the source tube to the wells with samples and mixed.

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.

The Second Strand Synthesis Mix 1 (**SS1**, 15 µl) is broadcasted to the wells with samples and mixed.

The machine stops and asks the operator to seal the sample plate with a film and do thermal treatment (Intervention #3: 98 °C for 1 minute, then cooling down slowly to 25°C, thermal ramp max. 0.5 °C/second). The thermally treated plate is to be placed on deck unsealed (after centrifugation) and the run is to be resumed.

The plate is incubated at 25 °C for 30 minutes. The **SS2 / E2** reagent (5 µl, prepared manually from the Second Strand Synthesis Mix 2, **SS2**, 4 µl, and the Enzyme Mix 2, **E2**, 1 µl) is transferred from the source tube to the wells with samples and mixed. The plate is incubated at 25 °C for 15 minutes.

Purification after Second Strand Synthesis

In this step the double-stranded library is purified using magnetic beads to remove all unnecessary reaction components.

Purification Beads (**PB**, 20 µl) are mixed in the source tube, dispensed to the sample wells, mixed, and incubated at 20 °C for 5 minutes. The plate is then placed on the magnet, the beads are separated for 5 minutes, and the supernatant is removed. The beads are resuspended in Elution Buffer (**EB**, 40 µl), mixed well and incubated at 20 °C for 2 minutes. Purification Solution (**PS**, 72 µl, or, optionally, 48 µl, for low RNA input) is added to the sample, mixed, and incubated again at 20 °C for 5 minutes. The plate is placed on the magnet, the beads are separated for 5 minutes, and the supernatant is removed. Each well is then washed with 120 µl Ethanol (80%) on the magnet. The Ethanol is removed after 30 seconds of incubation, and the Ethanol wash step is repeated once again. The beads are drying for 5 minutes to remove the rest of the Ethanol, and the plate is placed off magnet. Elution Buffer (**EB** 20 µl) is dispensed to each well, mixed, and incubated at 20 °C for 2 minutes. The plate is placed on magnet, the beads are separated for 5 minutes and the eluate (17 µl) is transferred to a clean plate. The PCR mix with enzyme (**PCR / E3**, 8 µl, prepared manually from the PCR mix, **PCR**, 7 µl, and the Enzyme Mix 3, **E3**, 1 µl) is transferred from the source tube to each well and mixed. The Barcodes (**BC**, 5 µl) from the Barcode Plate are also added and mixed. The Sample Plate is then moved to a position on deck easily accessible to the operator, and the robot stops. Phase 1 is finished.

Phase 2 – PCR Amplification

In this step the library is amplified to add the complete adapter sequences required for cluster generation and to generate sufficient material for quality control and sequencing.

The operator takes the plate out of the robot as instructed by the program, seals it with PCR foil, and places it in the thermocycler. Thermocycling is performed according to the specified program (see 7. Detailed Protocol, p.18). The plate is to be spun down after thermocycling.

Phase 3 – PostPCR

In this step the finished library is purified from PCR components that can interfere with quantification.

The PCR product (30 µl from each well) is purified using Purification Beads (**PB**, 30 µl), washed twice with 80% Ethanol, and then eluted in 20 µl of **EB**.

The Purification Beads (**PB**) are mixed in the source tubes, transferred (30 µl) to the plate with samples (containing 30 µl of amplified material), and mixed. The plate is incubated at room temperature for 10 minutes, and then placed on magnet, where the beads get separated for another 10 minutes. The supernatant (60 µl) is removed. Each well is then washed using 150 µl Ethanol (80%) on the magnet. The Ethanol is removed after 30 seconds of incubation, and the Ethanol wash step is repeated once again. The beads are dried for 5 minutes, the rest of the Ethanol is removed, and the plate is placed off magnet. Elution Buffer (**EB**, 20 µl) is dispensed to each well, mixed, and incubated at room temperature for 3 minutes. The plate is placed on magnet, the beads are separated for 3 minutes and the eluate (20 µl) is transferred to a clean plate. This plate is then moved to a position on deck easily accessible to the operator, and the robot stops.

Phase 3 and the whole autoQuantSeq protocol are finished.

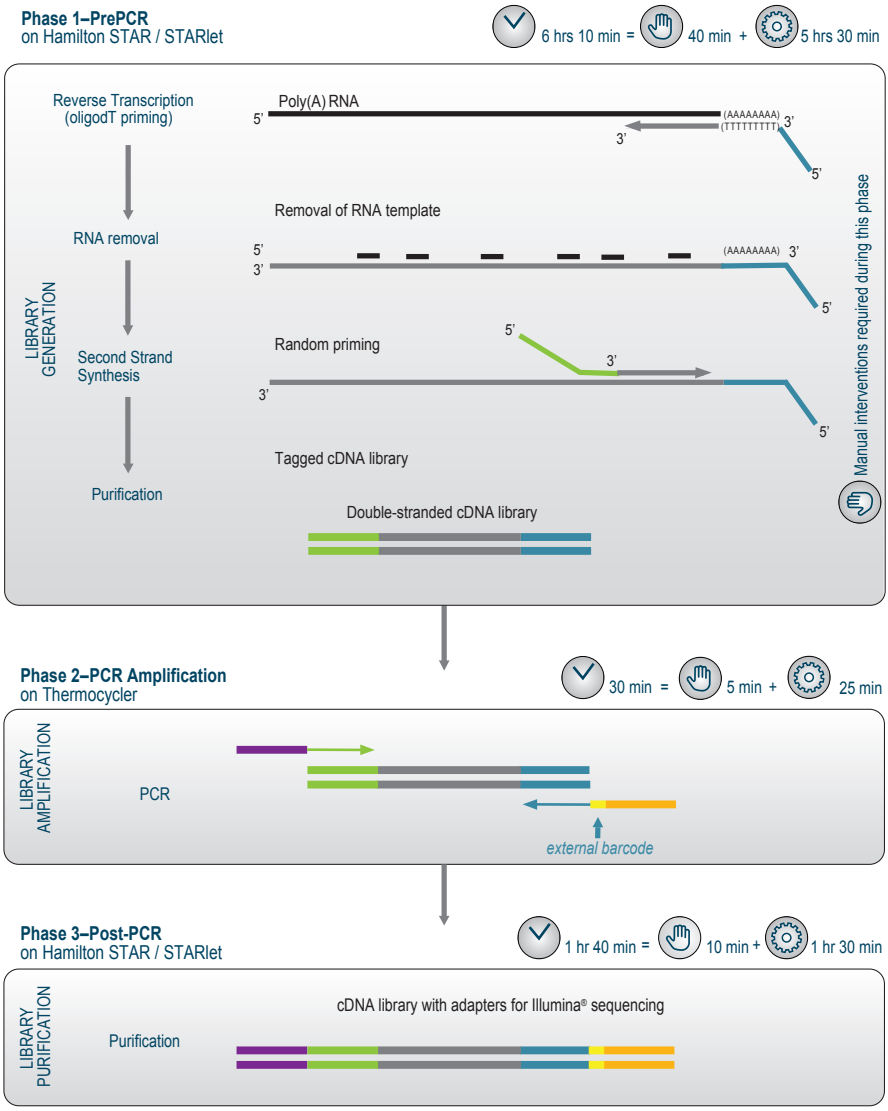


Figure 1. Schematic overview of the QuantSeq FWD library preparation workflow (Cat. No. 015). For QuantSeq REV (Cat. No. 016) the position of adapters for Read 1 (green) and Read 2 (blue) are switched. The execution times are given for 48 preps.

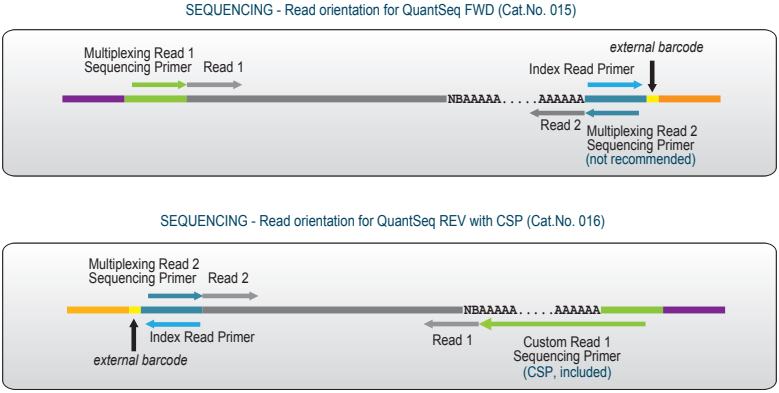


Figure 2. Sequencing Read orientation for QuantSeq FWD and QuantSeq REV. For QuantSeq FWD, Read 1 reflects the mRNA sequence. Paired-end sequencing is not recommended for QuantSeq FWD. QuantSeq REV is suitable for paired-end sequencing, and Read 1 reflects the cDNA sequence. A Custom Sequencing Primer (CSP, included in the kit) is required for Read 1.

3. Kit Components and Storage Conditions

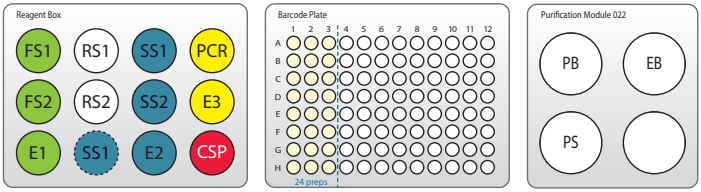


Figure 3. Location of kit components. For the 24 prep kit the dotted SS1 tube is missing and the barcode plate is only filled with set 1-3 (up to the blue dotted line). CSP (red lid color) is only required and included for QuantSeq REV (Cat. No. 016.24, Cat. No. 016.96, Cat. No. 016.2x96). All kits include Purification Modules.

Kit Component	Tube Label	Volume* provided for		Storage
		24 preps	96 preps	
First Strand cDNA Synthesis Mix 1	FS1 ●	132 µl	528 µl	-20 °C
First Strand cDNA Synthesis Mix 2	FS2 ●	250.8 µl	1003.2 µl	-20 °C
Enzyme Mix 1	E1 ●	13.2 µl	52.8 µl	-20 °C
RNA Removal Solution 1	RS1 ●	132 µl	528 µl	-20 °C
RNA Removal Solution 2	RS2 ●	132 µl	528 µl	-20 °C
Second Strand Synthesis Mix 1	SS1 ●	396 µl	1584 µl	-20 °C
Second Strand Synthesis Mix 2	SS2 ●	105.6 µl	422.4 µl	-20 °C
Enzyme Mix 2	E2 ●	26.4 µl	105.6 µl	-20 °C
PCR Mix	PCR ●	184.8µl	739.2 µl	-20 °C
Enzyme Mix 3	E3 ●	26.4 µl	105.6 µl	-20 °C
Barcode Plate (96-well plate)	BC	5 µl / reaction		-20 °C
Custom Sequencing Primer Version 2 (100 µM)**	CSP ●	25 µl	50 µl	-20 °C
Purification Module (Cat. No. 022) included in the kit				
Purification Beads	PB	1320 µl	5280 µl	+4 °C
Purification Solution	PS	2693 µl	10771 µl	+4 °C
Elution Buffer	EB	2904 µl	11616 µl	+4 °C

** only required for QuantSeq REV

* including a 10 % surplus

Upon receiving the QuantSeq kit, store the bottles **PB**, **PS**, and **EB** at +4 °C and the rest of the kit in a -20 °C freezer. Before use, check the contents of **PS**. If a precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

ATTENTION: The Custom Sequencing Primer (**CSP** ●) is only included and required for QuantSeq REV (Cat. No. 016) libraries. **CSP** ● has to be provided to the sequencing facility together with the lane mix. For further details on the usage of the CSP and the required volumes please consult Appendix F, p.33. Also forward this information to your sequencing facility before starting a sequencing run.

NOTE: The software components can be downloaded from www.lexogen.com (see p.16).

4. User-supplied Consumables and Equipment

Reagents

Reagent	Supplier
80% fresh EtOH (about 31 ml for 48 preps)	Various
Elution Buffer (10mM TRIS, pH 8.0, about 6 ml for 48 preps)	Various
(optional) SYBR Green I	Sigma-Aldrich, Cat.No. S9430

General Laboratory Equipment and Supplies

Equipment	Supplier
Suitable pipette tips (tips with aerosol barriers recommended)	Various
Thermocycler (Bio-Rad HSP-96 PCR-plate compatible)	Various
Microfuge	Various
Vortexer	Various
Microplate centrifuge	Various
Plate seals	Various

Optional Equipment and Material (for RNA quantification and quality control)

Equipment and Material	Supplier
UV-spectrophotometer	Various
Automated microfluidic electrophoresis station	Agilent Technologies 2100 Bioanalyzer, PerkinElmer LabChip GX II
qPCR machine and library standards	Various
Benchtop fluorometer and appropriate assays	Various
Agarose gels, dyes, and electrophoresis rig	Various

The complete set of material and labware necessary for RNA extraction and quality control is not listed. Consult Appendix A (p.25) for more information on RNA quality. Consult Appendix D (p.30) for information on library quantification methods.

Hamilton Microlab STAR Requirements

The protocol can be installed on a Hamilton Microlab STAR or STARlet system which has been hardware-configured to run NGS library preps. The hardware and software pre-configuration is identical to that of the automated library preparation using NEBNext Ultra DNA Library Prep kit for Illumina (E7370), implemented by Hamilton Robotics GmbH. Ask Lexogen for installation details.

On-deck Consumables

Consumable	Description	Original Vendor and Part No.	No. Used per Run (48 samples, FULL)
Reagent tubes	Microtube 1.5 ml, PP	SARSTEDT 72.692.005	13
PCR Plates	Bio-rad 96-well full skirt hard-shell plate, or 4titude FrameStar 96-well skirted PCR plate	Bio-Rad HSP-9631 4ti-0960	2
Reagent container	Container 50 ml	Hamilton 56694-01	3
Lids	946 Lid–universal, robotic friendly, PS	Seahorse Bioscience 200856-100	2
Tips 1000 µl	Conductive CO-RE Tips High Volume, with filter, sterilized	Hamilton 235940	3
Tips 300 µl	Conductive CO-RE Tips Standard Volume, with filter, sterilized	Hamilton 235938	41
Tips 50 µl	Conductive CO-RE Tips 50 µl, with filter, sterilized	Hamilton 235979	864

5. General Guidelines

Liquid Handler Usage

- Always wear gloves when touching the liquid handler casing, the deck, the Inheco controller, and any other parts of the robotic workstation.
- Always wear gloves when touching any consumables on deck.
- When placing the consumables on the deck, make sure they are securely positioned.
- Do not use consumable types other than specified in this document, even if they seem to be very similar to the specification. The liquid handler is programmed to the dimensions and specifications of the consumables mentioned and even minor physical differences may cause malfunction of the handler. Ask Lexogen if in doubt.

RNA Handling During Run Preparation

- 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. Before starting a library preparation, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) according to the manufacturer's instructions.
- 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 the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

Manual Bead Handling

- Beads are stored at +4 °C and must 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. Avoid centrifuging of beads.
- 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 gentle vortexing if this occurs with your tips.

Pipetting and Handling of (Viscous) Solutions

- Enzyme mixes, **PS**, **PB**, and **SS1** 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.

Miscellaneous

- Ensure that adequate volumes of all reagents and the necessary equipment is available and set to the proper temperatures 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 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 right before use or store in a -20 °C benchtop cooler.
- The step requiring a thermocycler has 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.
- To keep track of the pipetting steps, tick the respective cells in the worksheet indicating a completed column of a plate.
- Inspect each plate and tube after you have finished pipetting. If bubbles are present in the liquids, spin the plate shortly. Protect the content of the plate with a lid when spinning.
- Unless explicitly mentioned otherwise, all handling steps have to be carried out at room temperature. Make sure to set the centrifuge to room temperature when spinning.
- Label each plate and tube immediately after preparation using a lab marker. To enable easy identification on the deck, we recommend to mark the plate in the upper left corner on the top surface.

6. Software Download and Installation

NOTE: *Installing the application on the instrument for the first time requires some experience and skills. Therefore, we recommend asking for Lexogen support. The following description is just for your information. More detailed instructions can be found in the Installation Manual which is included in the software distribution package (* www.lexogen.com *).*

Prerequisites

The library protocol using NEBNext Ultra Library Prep Kit for Illumina (E7370), implemented by Hamilton Robotics GmbH, has to be installed on the system. The autoQuantSeq protocol makes use of some software components (libraries, liquid class definitions, labware definitions, configuration tools) of the NEBNext protocol.

Download

All software components of the autoQuantSeq kit can be downloaded from Lexogen website at www.lexogen.com. Please download the latest available release and always read the respective Release Notes file included which may supersede the instructions given in this manual.

Installation

1. Unzip the LEX_autoQuanTseq_YYMMDDn.zip package in a temporary folder.
2. Copy the contents of the *Methods_Lexogen_autoQuantSeq* to (typically)
   C:\Program Files\HAMILTON\Methods\Lexogen\autoQuantSeq
   (If the folder already exists, delete its contents completely before copying).
3. Copy the contents of the *Library_Lexogen_autoQuantSeq* to (typically)
   C:\Program Files\HAMILTON\Library\Lexogen\autoQuantSeq
   (If the folder already exists, delete its contents completely before copying).
4. Update the Liquid Class Definition Database by using the Hamilton CO-RE Liquid Editor and importing all liquid classes listed in the *LEX_Liquids.mdb* (if entries with same names already exist in the database, overwrite them).
5. Install desktop shortcut: Copy the file *RUN_QuantSeq.lnk* to your desktop.

Dry and Wet Tests

After installing the application, we recommend to run a dry test and a wet test first. The dry test will ensure that gripper transport of plates, tips, and lids is robust, and there are no collisions with accessories and consumables. It also ensures the liquid handling is clean and the beads are not lost during cleanups.

The wet test can be done using a kit with dummy liquids for wet testing available from Lexogen (Cat. No. 019.24).

7. Detailed Protocol

Read all the instructions carefully prior to beginning the run. This description assumes the Application is already installed on the control PC of the Microlab STAR.

7.1 First Preparation Steps

Purification Beads (**PB**) and Purification Solution (**PS**) need to be equilibrated to room temperature for about 30 minutes before use. They may be taken out of 4 °C storage before beginning.

7.2 Workbook Preparation

Open the Microsoft Excel workbook in this filepath of the Hamilton Microlab STAR/STARlet control PC:

C:\Program Files\HAMILTON\Methods\Lexogen\autoQuantSeq\
autoQUANTSEQ STAR Library Prep Workbook.xls

In the *Reagent Prep & Fill Scheme* worksheet of this workbook, you will find detailed pipetting schemes as well as reagent preparation guidelines. Fill the yellow marked cells in the header of the Reagent worksheet (assay ID, date, number of samples etc.). All the pipetting schemes and reagent preparation recipes will be automatically adapted following your input. Do not modify any other content of the worksheet manually, except for the yellow-marked cells.

The **Assay ID** and **Date** entries do not affect the run in any way and are for reference only.

The **Protocol variant** entry specifies which phases of the protocol should be performed by the robot. You can select between **PrePCR only**, **PostPCR only**, or **FULL** (i.e., both PrePCR and PostPCR in a single run).

For the **Number of samples**, you can select any number from 1 to 48.

In the **First barcode well in P1** entries (one for row letter, one for column number), you specify the mutual correspondence between barcodes and samples. Refer to p. 21 for explanation.

In the **Sample Overview** table below, you can identify your samples by an **ID** and a **Note**. Both entries are optional and do not affect the run in any way.

Denaturation Step Option

In the **Apply denaturation step:** cell, select between **YES** (standard protocol, RNA denaturation step will be applied), or **NO** (the denaturation option will be skipped; this is recommended for low quality RNA or FFPE samples, see Appendix C, p.29).

RNA Input Quantity Option

In the **RNA input quantity:** cell, select between **more than 10ng** (standard protocol), or **10ng and below** (in this case, 48 µl of **PS** will be used in the purification after second strand synthesis, instead of 72 µl for standard protocol).

Save the modified Microsoft Excel workbook under the original name in the original filepath. It is recommended to print out the respective worksheets at this stage, and use the printout as your reagent preparation guide. For a routine work, you may find helpful to print out just the Short Preparation Guide (the *Short Guide* worksheet of the workbook), which contains all necessary information for run preparation in a concise single-sheet format.

The workbook will also show you labware consumption and the estimated execution time for this run.

7.3 Thermocycler Programming

We recommend to set up the programs for the thermocycler in advance. Four different thermal procedures are required during the run. At the given step of the protocol, the application will prompt for the particular manual intervention, which includes plate sealing, thermal treatment on the thermocycler, optional spinning, plate unsealing, and resuming the run.

The thermal procedures required are summarized in the table below.

Intervention Step	Thermal Procedure
Incubation #1	• 85 °C, 3 minutes • 42 °C ∞ Volume 10 µl, lid temperature 105 °C
Incubation #1A	• 42 °C, 15 minutes • 42 °C ∞ Volume 20 µl, lid temperature 105 °C
Incubation #2	• 95 °C for 10 minutes • 25 °C ∞ Volume 25 µl, lid temperature 105 °C
Incubation #3	• 98 °C for 1 minute • cool down to 25 °C with 0.5 °C/sec ramp • 25 °C ∞ Volume 45 µl, lid temperature 105 °C

PCR	• 98 °C for 1 minute
	Repeat 13 times (refer to p.28 for more details on cycle number specification):
	  • 98 °C for 10 seconds
	  • 65 °C for 20 seconds
	  • 72 °C for 30 seconds
	• 72 °C for 1 minute
	• 10 °C ∞
	Volume 30 µl, lid temperature 105 °C

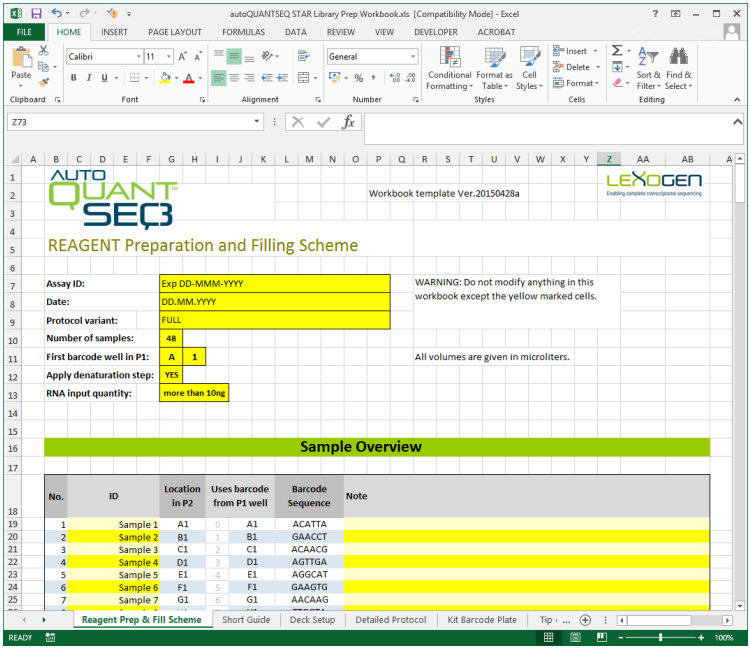


Figure 4: A fragment of the autoQuantSeq workbook. Note that the actual layout of the spreadsheet in the software distribution may differ slightly from the layout shown.

7.4 Reagent Preparation

Reagents in Tubes and Troughs

The detailed setup in described in the Microsoft Excel workbook. The *Reagent Prep & Fill Scheme* worksheet contains recipes for master mixes used in the run. Follow the work sequence given in the tables, starting from high-volume reagents, and finishing with samples.

Pay special attention to homogenization of the Purification Beads (**PB**) prior to filling them into the tubes.

Note on Elution Buffer

To simplify the manual preparation, the Elution Buffer (**EB**) is presented in a 50 ml reservoir (trough). Robotic aspiration from this type of vessel requires a higher dead volume. Therefore, the amount of the **EB** required for automation might exceed the amount delivered in the kit. In such situation, the **EB** can simply be substituted by **10 mM TRIS pH 8.0**.

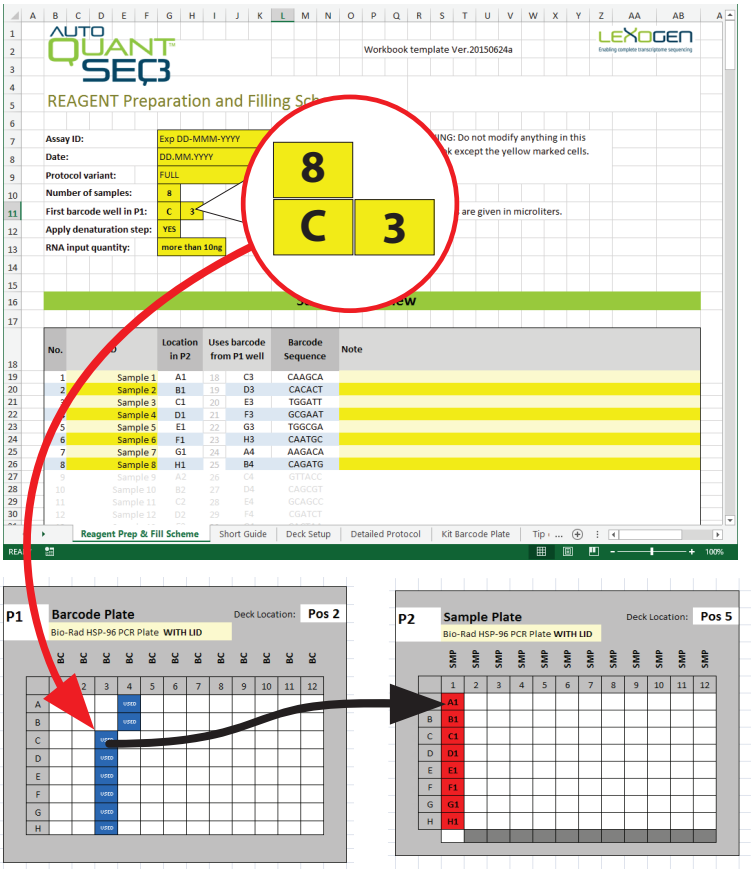


Figure 5: Assignment of the barcodes to the samples.

P1 Plate – Barcodes

The barcodes come prefilled in the Kit Barcode Plate, which is used as the P1 Plate in the protocol. The Kit Barcode Plate can be reused several times until all barcodes are used up. The correspondence of the samples to the respective barcode positions in the P1 Plate has to be specified in the workbook. The samples are always filled in starting from the A1 well, and following the sequence A1, B1, C1, …, H1, A2, B2, … etc. The corresponding barcodes also follow this sequence, but can start at a different well of P1. This starting well is specified in the **First barcode well in P1** entry in the workbook. An example of the barcode-to-sample correspondence is given in Figure 5.

The Barcode Plate has to be stored at -20 °C, sealed with a cap stripes and covered with a lid. Before the run, thaw the plate, spin down carefully, remove the cap stripes from the wells you

want to use in this run (you have to remove the whole stripe columns), cover the plate with the lid again, and place it on deck. After the run, cover the plate with a lid again and refreeze. We recommend using the Kit Barcode Plate for up to four runs.

The filling scheme of the Kit Barcode Plate with barcode sequences can be found on p.32.

P2 Plate – Samples

The P2 Plate contains input RNA samples. Make sure you pipette the correct volumes. Spin down the plate, if necessary, to collect all the reagent liquid at the bottom of the tube. Cover the plate with a lid prior to placing it on deck.

7.5 Starting the Application

Start the application by double-clicking the autoQuantSeq STAR icon on your desktop (Figure 6).

Figure 6: The autoQuantSeq icon on the desktop..

The application will present itself by a protocol progress window in the lower right corner of the screen and with an introductory window with a logo. Follow the instructions given in the windows.

7.6 Deck Setup

The deck setup for the specified number of samples and the given protocol variant is shown in the workbook. Moreover, an adaptive display of the actual deck setup is shown in one of the windows at the beginning of the protocol (Figure 7). The user has to tick the check boxes shown one after another, to confirm that the corresponding labware with reagents has been placed on deck. The Continue button is only shown after all check boxes have been checked.

The operator also has to provide the tips in the locations and numbers indicated. The setup of the tips is done in a standard tip loading dialog of the Hamilton control software.

7.7 Phase 1 – PrePCR Run

During the run, several manual interventions will be necessary, such as sealing or unsealing a plate, and/or treating the plate thermally on an external thermocycler. Detailed instructions are given on the application panel (see an example in Figure 8). Follow them carefully. For thermal

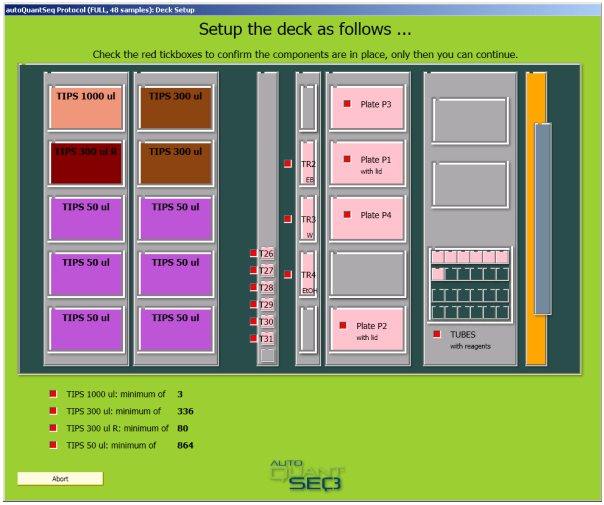


Figure 7: The Deck setup dialog.

procedures, you may use the programs stored in the thermocycler (see p.19). After each manual intervention, click the Continue button to resume the run. You will be informed about the approximate walk-away time to the next intervention.

Note that immediately after some of the interventions you may be asked to recharge the tips on deck (a standard tip loading dialog of the Hamilton control software will be shown).

When the PrePCR phase is complete, the plate is prepared for the PCR amplification.

If you chose the **PrePCR only** protocol variant, the liquid handler will now stop and the protocol is finished.

7.7 Phase 2 – PCR Amplification

Take the plate from the deck and seal it with a film. If bubbles are present in the liquid, spin the plate shortly. Place the plate on a thermocycler. The recommended library amplification

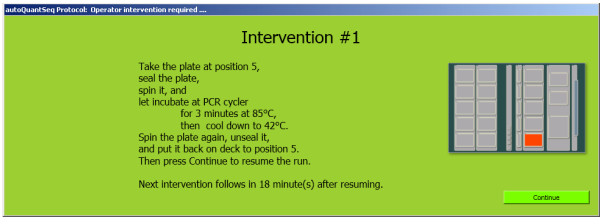


Figure 8: The Intervention request dialog.

program is given in the table on p.19. Refer to p.28 for information regarding optimization of library amplification.

7.8 Phase 3 – PostPCR Run

If you chose the **PostPCR only** protocol variant, the run will only consist of this phase, which then begins with a series of introductory and setup dialogs as described above. If you chose the **FULL** protocol variant, the run simply continues.

No manual intervention is required during this protocol phase.

When the PostPCR phase is complete, the final plate contains amplified and purified libraries. Discard used tubes and reservoirs. Empty the tip waste.

The autoQuantseq application is completed.

8. Appendix A: RNA Requirements

RNA Amount and Quality

In general, high quality mRNA-Seq data relies on high quality input RNA. However, as QuantSeq is a 3' mRNA-Seq protocol the quality of the RNA input is not as critical as for other RNA-Seq applications. With QuantSeq even lower quality RNA can yield good RNA-Seq results, therefore QuantSeq is highly suitable for FFPE samples. For further details on FFPE samples refer to Appendix C, p.29.

The amount of total RNA required for QuantSeq depends on the poly(A) RNA content of the sample in question. This protocol was tested extensively with various cell cultures, mouse and plant tissues, yeast, fungi, and human reference RNA (Universal Human Reference RNA (UHRR) and Brain Reference RNA). Typical inputs of 500 ng total RNA generate high quality libraries for single-end 50 nt (SR50) or paired-end 100 nt sequencing (PE100) with 12 cycles of library amplification. For mRNA-rich tissues (such as kidney, liver, and brain) input RNA may be decreased to 50 ng without adjusting the protocol. Lower RNA inputs (500 pg–10 ng) require protocol adjustments (see Appendix B, p.28).

With reduced total RNA input cycle numbers need to be adjusted accordingly (see Appendix B, p.28). For tissues with lower mRNA content (such as lung and heart) we recommend using 500 ng total RNA input. Low RNA input increases the likelihood of linker-linker artifacts and for samples using less than 5 ng input RNA an additional clean up of the lane mix might be required.

The input requirements for your particular experiment may be different, as RNAs differ in their mRNA content. If RNA input is not sufficient, either due to naturally low poly(A) RNA content or degraded RNA, additional cycles of library amplification may be necessary. Overcycling of libraries – indicated by a second high molecular weight peak between 1000–9000 bp in a Bioanalyzer trace – should be prevented as this may lead to distortions in transcript abundance and library quantification.

As a starting point, we recommend performing the protocol initially with 500 ng total RNA.

Lexogen also offers a PCR Add-on Kit for Illumina (Cat. No. 020.96), which can be used for qPCR assays, should you need to determine exact cycle numbers for your endpoint PCRs.

qPCR to Determine the Exact cycle Number of Your Enpoint PCRs

For determining the cycle number of your endpoint PCR we recommend to make a run with (as an example) 4 samples, each with 500 ng or 2 µg of total RNA according to the expected poly(A) content. Use a new barcode plate filling 8.5 µl of Barcode 00 (**BC00** ●, included in the PCR Add-on Kit for Illumina, Cat. No. 020.96) to the used wells of the first column of the P5 plate. Run the Phase1-PrePCR phase of the protocol. Then add SYBR Green I (or an equivalent fluorophore) to the PCR reaction to a final concentration of 0.1x. For 0.1x SYBR Green I add 1.2 µl 2.5x SYBR Green I solution (1:4000 SYBR Green I dilution, diluted in DMSO) to each reaction. The total

PCR reaction volume will be 31.2 µl. SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines has to be adjusted manually. Overcycle this initial qPCR (30 cycles or even more if little input material was used) and then determine the fluorescence value at which the fluorescence reaches a plateau. Calculate where the fluorescence is 33 % of the maximum, and this is the cycle number you should use for the endpoint PCR. There is no need to purify or analyze the overcycled PCR reaction on a Bioanalyzer.

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.

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 much more intensely 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. 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 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 Variant Control Mixes, Cat. No. 025.03), to serve as a control and anchor set for the comparison of RNA-Seq experiments. The SIRV sequences enable bioinformatic algorithms to accurately map, assemble, and quantify isoforms, and provide the means for the validation of these algorithms in conjunction with the preceding RNA-Seq pipelines. The SIRVs consist of 69 artificial RNA transcripts with no identity to any known genomic sequences hence they can be spiked into any RNA. Each SIRVs box contains 3 tubes labeled E0, E1, or E2. Each mix contains all 69 SIRV transcripts, but at different molar concentrations to each other. SIRV Mixes can be used as single spike-ins, or as a combination of two or three different SIRV mixes for the assessment of differential gene expression.

ERCC RNA Spike-In Controls

To enable the hypothesis-neutral calculation of strandedness, to assess internal oligodT priming events, and as a true reference on detection limit and preservation of dynamic range, we highly recommend the addition of artificial transcripts of known strand orientation and concentration such as the ERCC RNA Spike-In Controls (Ambion Inc.). These sets of RNAs 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 as internal priming events of the oligodT primer, when in fact those are true 3' ends. As ERCC transcripts only have one defined 3' end, this provides the only true measure to determine internal priming.

9. Appendix B: PCR Cycles – Low Input Material

Typically we recommend using 500 ng total RNA input as starting material. If not enough total RNA is available, the input RNA amount can be reduced. The table below depicts some examples of libraries synthesized with the QuantSeq kit using different input RNA amounts.

Set the **RNA input quantity** cell in the workbook appropriately. The option **10 ng and below** reduces the volume of **PS** added during purification after second strand synthesis from 72 µl to 48 µl.

The table below comprises some results obtained from using different amounts of total RNA input. Reference values were generated using Universal Human Reference RNA (UHRR). Other RNAs with lower mRNA content may require more PCR cycles. Please also refer to the QuantSeq Frequently Asked Questions (FAQs) page at www.lexogen.com for cycle number recommendations for some other RNAs (e.g., different mouse tissues, plants, or yeast). Avoid overcycling (indicated by a second high molecular weight peak between 1000 - 9000 bp in a Bioanalyzer trace). Take advantage PCR Add-On Kit (Cat. No. 020.96) and the qPCR assay as described on p.25, if you are uncertain about the cycle number for your endpoint PCR.

Input RNA (UHRR)	PS used	Library*			Insert				Library yield		PCR cycles
		Start [bp]	End [bp]	Mean size*	Mean size	≥ 50 nt	≥ 100 nt	≥ 200 nt	ng/µl	nM	
500 ng	72 µl	122	1000	308	186	93 %	60 %	17 %	6.0	34.8	13
50 ng	72 µl	122	1000	318	196	93 %	67 %	23 %	3.1	17.0	17
10 ng	48 µl	122	1000	350	228	95 %	77 %	32 %	2.1	10.7	20
5 ng	48 µl	122	1000	292	170	94 %	66 %	17 %	2.1	12.1	21

*All libraries are prepared with external Barcodes. Linker sequences are 122 bp including the 6 nt long external Barcodes.

10. Appendix C: 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, it is recommended to skip the denaturation step. This can be done simply by selecting **NO** in the **Apply denaturation step:** cell of the Workbook. We also recommend to reduce the volume of **PS** added during purification after second strand synthesis from 72 µl to 48 µl, which can be done in the workbook by setting the **RNA input quantity** to **10 ng and below**.

As the RNA amount is often a limiting factor with FFPE samples, QuantSeq was tested with 10 ng – 50 ng FFPE or degraded RNA input.

The table below comprises some results obtained from using different amounts of FFPE RNA input with a RIN of 2.8 (DV200 of 87 %) (manual processing). The DV200 value is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV200, the more degraded the RNA is. Other FFPE RNAs or RNAs with lower DV200 values may require more PCR cycles, hence we would strongly recommend using the PCR Add-On Kit (Cat. No. 020.96) and taking advantage of the qPCR assay as described on p.25.

Input RNA (UHRR)	PS used in step 17	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	48 µl	122	500	220	98	56 %	12 %	1 %	1.5	12.3	18
10 ng	48 µl	122	500	233	111	84 %	36 %	3 %	1.2	8.7	24

*All libraries are prepared with external Barcodes. Linker sequences are 122 bp including the 6 nt long external Barcodes.

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

11. Appendix D: 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 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 LabChip GX II (Perkin Elmer) or 2200 TapeStation (Agilent Technologies, Inc.) are recommended. Typically, 1 µl of a QuantSeq library produced according to the directions in this manual is sufficient for analysis. Depending on the minimum sample loading requirements for each instrument, 1 µl of the finished library may be diluted to the required volume (e.g., 2 µl sample for TapeStation and 10 µl for LabChip GX II).

More accurate library quantification can be achieved with custom or commercially available qPCR assays. With these assays, the relative or absolute abundance of amplifiable fragments contained in a finished QuantSeq library is calculated by comparing Cq values to a set of known standards. While generating 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 discernable from the actual library in the qPCR assay as both will be amplified. Hence the use of such an assay for quantification in combination with microcapillary electrophoresis analysis for size distribution is highly recommended.

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. Most UV-Vis spectrophotometers are not sensitive enough at these concentrations to accurately quantify NGS libraries and should be avoided.

Typical Results

Typical concentrations are between 8 to 15 nM (1.4 to 2.7 ng/µl) for 50 ng (15 cycles) and 500 ng (12 cycles) input RNA, respectively, with most inserts between 80 to 150 nts in length (see also Appendix B, p.28). The variability, σ/µ or SD/AVG, of the protocol itself stands below 25%, a plausible value in the light of DNA analytics variability (specifications LabChip HT DNA High Sensitivity assay: accuracy ±30%, precision 20% CV).

A peak at ~81 bp is a primer artifact which will not bind to the surface during cluster formation.

For examples of expected NGS library parameters see Figure 9 (amplification in 13 PCR cycles, elution in 20 µl, and analysis by a capillary electrophoresis instrument).

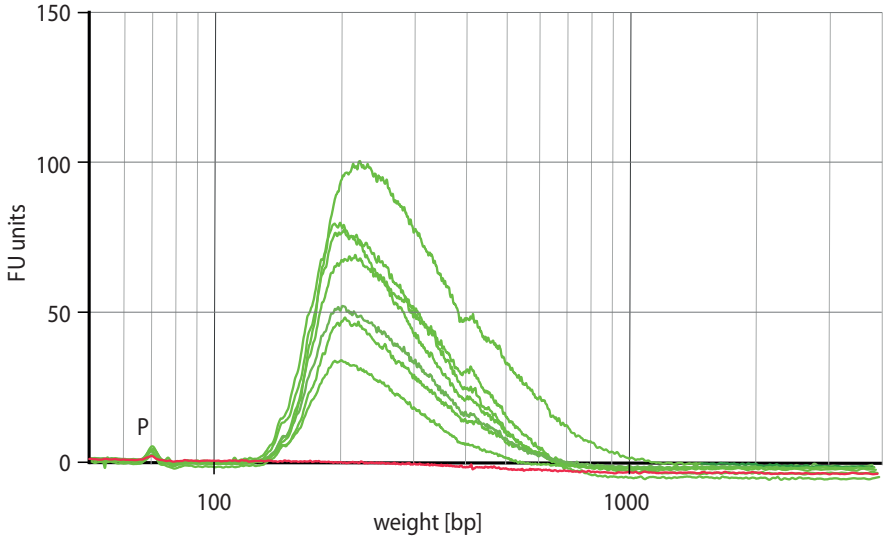


Figure 9: Electrophoretic traces of autoQuantSeq NGS libraries. 48 NGS libraries were generated, including 6 negative samples. 8 randomly selected libraries including one library produced from a negative sample (shown in red) were analyzed by a Bioanalyzer electrophoresis instrument with a High Sensitivity DNA Kit. For positive samples. Identical starting material of 500 ng Agilent's Universal Human Reference RNA (UHR-RNA) each produced within 13 PCR cycles 18 µl libraries of 13.2 ± 5.4 nM, or 1.70 ± 0.76 ng/µl, with a total average length of 230.3 ± 16.4 bp corresponding to 108.3 bp insert length due to adaptor sequences with a length of together 122 bp. P are primer-derived side-products which do not affect sequencing. Values given as AVG ± SD.

12. Appendix E: Multiplexing

QuantSeq libraries are designed for a high degree of multiplexing. External barcodes are introduced during the PCR amplification step.

External Barcodes

External Barcodes allowing up to 96 samples to be sequenced per lane on an Illumina flow cell are included in the kit in the Barcode Plate (**BC**). External Barcodes are 6 nt long and require an additional index-specific sequencing reaction.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC01: ACATTA	BC02: GGTGAG	BC03: CGAAGG	BC04: AAGACA	BC05: TAATCG	BC06: CGCAAC	BC07: AATAGC	BC08: TTAACT	BC09: AATGAA	BC10: GATTGT	BC11: ATAAGA	BC12: GCCACA
B	BC13: GAACCT	BC14: CGGTTA	BC15: AACGCC	BC16: CAGATG	BC17: GATCAC	BC18: CGCGGA	BC19: CCTAAG	BC20: GGCTGC	BC21: ACCAGT	BC22: GTGCCA	BC23: AGATAG	BC24: TCGAGG
C	BC25: ACAACG	BC26: GCGCTG	BC27: CAAGCA	BC28: GTTACC	BC29: CTCTCG	BC30: CCAATT	BC31: TTCGAG	BC32: CGTCGC	BC33: TGTGCA	BC34: ACCGTG	BC35: ATACTG	BC36: ATGAAC
D	BC37: AGTTGA	BC38: GACGAT	BC39: CACACT	BC40: CAGCGT	BC41: TGCTAT	BC42: TCTTAA	BC43: CCGCAA	BC44: CTCCAT	BC45: GTCAGG	BC46: ACGTCT	BC47: GAGTCC	BC48: GACATC
E	BC49: AGGCAT	BC50: ACCTAC	BC51: TGGATT	BC52: GCAGCC	BC53: CGCCTG	BC54: CCGACC	BC55: TATGTC	BC56: TGACAC	BC57: ACAGAT	BC58: AGACCA	BC59: GCTCGA	BC60: ATGGCG
F	BC61: GAAGTG	BC62: AGAATC	BC63: GCGAAT	BC64: CGATCT	BC65: CATCTA	BC66: AAGTGG	BC67: TGCACG	BC68: TCGTTC	BC69: ACACGC	BC70: GTAGAA	BC71: AGTACT	BC72: GCATGG
G	BC73: AACAAG	BC74: AACCGA	BC75: TGGCGA	BC76: CACTAA	BC77: AAGCTC	BC78: TACCTT	BC79: CTAGTC	BC80: AATCCG	BC81: GTGTAG	BC82: ACTCTT	BC83: TCAGGA	BC84: ATTGGT
H	BC85: TTGGTA	BC86: CAACAG	BC87: CAATGC	BC88: GGAGGT	BC89: CAGGAC	BC90: GGCCAA	BC91: CTCATA	BC92: CCTGCT	BC93: GGTATA	BC94: TTCCGC	BC95: TAGGCT	BC96: ATATCC

External barcode sequences are available for download at www.lexogen.com, and are also available in the workbook. QuantSeq is specifically designed for multiplexing 48 or 96 samples per sequencing lane (depending on the intended read depth). Note that the automated protocol only allows preparing up to 48 samples per run.

The 24 reaction QuantSeq kits (Cat. No. 015.24, Cat. No. 016.24) include Barcode Set 1 (BC01/BC13/BC25/BC37/BC49/BC61/BC73, and BC85), Barcode Set 2 (BC02/BC14/BC26/BC38/BC50/BC62/BC74, and BC86), and Barcode Set 3 (BC03/BC15/BC27/BC39/BC51/BC63/BC75, and BC87).

The 96 reaction kits (Cat. No. 015.96, Cat. No. 015.2x96, Cat. No. 016.96, Cat. No. 016.2x96) include all Barcode Sets (Set 1 - 12) and here Barcodes can be combined across rows (Set A: BC01–12, Set B: BC13–24, and so on) or columns (Set 1–12).

In general, we recommend processing a minimum of 8 samples, better 12 at a time and using a complete set of 8 or 12 Barcodes for multiplexing (e.g., Set 1 or Set A if a 96 reaction kit is used, respectively). However, if fewer Barcodes are required care should be taken to always use sets of Barcodes which give a signal in both lasers (red and green channels) for each nucleotide position. Sets 1–12 and A–H fulfill these criteria. The individual libraries within a lane should be mixed at an equimolar ratio to ensure this balance.

13. Appendix F: Sequencing*

General

The amount of library loaded onto the flowcell will greatly influence the number of clusters generated. Each sequencing facility has slightly different preferences of how much to load. From our experience a good starting point is to load between 7 and 14 pM (pmol/l) of a QuantSeq library onto the flowcell.

For paired-end sequencing we recommend using QuantSeq REV (Cat. No. 016.24, 016.96).

A schematic representation of the two types of QuantSeq libraries (FWD Cat. No. 015 and REV Cat. No. 016) is shown below. The required sequencing primers are also listed.

QuantSeq FWD Libraries with External Barcodes (Cat. No. 015.24, Cat. No. 015.96, Cat. No. 015.2x96)

External Barcodes (6 nt) are introduced during PCR.
For QuantSeq FWD libraries, Read 1directly corresponds to the mRNA sequence.

```
                              5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert…
3'TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert…

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

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

5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

Index Read: Multiplexing Index Read Sequencing Primer (not supplied):

5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied):

5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

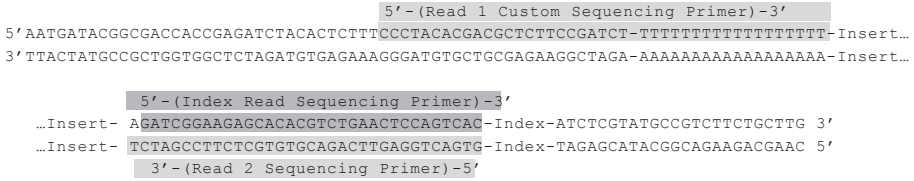
ATTENTION: We do not recommend paired-end sequencing for QuantSeq FWD (Cat.No. 015.24, Cat. No. 0.15.96, Cat. No. 0.15.2x96), as the quality of Read 2 would be very low due to the poly(T) stretch at the beginning of Read 2.

For paired-end sequencing please use QuantSeq REV (Cat.No. 016.24, Cat. No. 016.96, Cat. No. 016.2x96).

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

QuantSeq REV Libraries with External Barcodes (Cat. No. 016.24, Cat. No. 016.96, Cat. No. 016.2x96)

External Barcodes (6 nt) are introduced during PCR.
For QuantSeq REV libraries, Read 1 corresponds to the cDNA sequence.



Read 1: Custom Sequencing Primer (included):

5' CCCTACACGACGCTCTTCCGATCTTTTTTTTTTTTTTTTTT 3'

ATTENTION: Do not use Multiplex Read 1 Sequencing Primer for QuantSeq REV (Cat.No. 016.24, Cat. No. 0.16.96, Cat. No. 0.16.2x96). Multiplex Read 1 Sequencing primer would result in a failed sequencing run as cluster calling would be impossible due to the poly(T) stretch.

ATTENTION: Do not mix CSP and Read 1 Sequencing Primer! A primer mixture would result in low clusters calls and the resulting reads would be contaminated by poly(T) stretches.

Index Read: Multiplexing Index Read Sequencing Primer (not supplied):

5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied):

5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Usage of the Custom Sequencing Primer CSP ●

For QuantSeq REV (Cat. No. 016.24, Cat. No. 016.96, Cat. No. 016.2x96) the Read 1 linker sequence is located at the 5' end of the oligodT primer. Here a Custom Sequencing Primer (**CSP ●** Version 2, included in the kit) is required for Read 1. The Custom Sequencing Primer covers the poly(T) stretch. Without the Custom Sequencing Primer cluster calling is not possible.

ATTENTION: Do not mix CSP ● and Read 1 Sequencing Primer! Do not mix **CSP ●** into HP10! A primer mixture would result in low clusters calls and the resulting reads would be contaminated by poly(T) stretches.

HiSeq 2000, HiSeq 2500 (CSP ● Version 2 added on cBot)

CSP Version 2 should be provided in a tube strip at 0.5 µM final concentration in a volume of 120 µl (final concentration 0.5 µM, to be diluted in HT1 = Hybridization buffer). Take 0.6 µl of 100 µM **CSP** Version 2 and add 119.4 µl of HT1 buffer per sequencing lane. Place the 8-tube strip into the cBot position labeled primers.

HiSeq 2500 (CSP ● Version 2 replaces HP10 in cBot Cluster Generation Reagent Plate)

Alternatively, **CSP** ● Version 2 can be placed directly into the cBot Cluster Generation Reagent Plate. **ATTENTION:** The standard Illumina Multiplex Read 1 Sequencing Primer solution HP10 (for V4 chemistry located in row 2) provided in the cBot Cluster Generation Reagent Plate has to be **REMOVED** first! The Illumina V4 chemistry cBot Cluster Generation Reagent Plate only has 8 rows filled. A simple trick is to have the empty rows facing towards you, this way if you want to use a **CSP** ● in lane 1, you have to remove the HP10 solution from well 1 (first one on the far left) of the 2nd row, rinse the well a couple of times with HT1 and then add the diluted **CSP** ● Version 2. For this take 1.25 µl of 100 µM **CSP** ● Version 2 and add 248.75 µl of HT1 buffer per sequencing lane. The **CSP** ● should be at 0.5 µM final concentration in a volume of 250 µl (final concentration 0.5 µM, to be diluted in HT1 = Hybridization buffer). **ATTENTION:** Do not add the CSP to the Standard Illumina Multiplex Read 1 Sequencing Primer = HP10 solution! Always use fresh HT1 and add the **CSP** ● / HT1 dilution to the empty and rinsed well.

HiSeq 2500 - Rapid Run

Add 12.5 µl of 100 µM **CSP** ● Version 2 to 2487.5 µl HT1 = Hybridization buffer, resulting in a total volume of 2.5 ml and a final **CSP** ● concentration of 0.5 µM. In a rapid run, both lanes will use the same sequencing primer. It is not possible to run the two lanes with different sequencing primers.

MiSeq

Clustering is performed on the machine, not on the c-Bot. The MiSeq uses a reservoir of 600 µl with 0.5 µM sequencing primer final concentration, i.e., 3 µl of 100 µM **CSP** ● Version 2 in 597 µl HT1.

HiSeq 3000, HiSeq 4000 (CSP ● Version 2 replaces HP10 in cBot Cluster Generation Reagent Plate)

Usage of a custom sequencing primer is currently not supported on HiSeq 3000 and 4000 machines. A work around as described for the HiSeq2500 (**CSP** ● Version 2 **REPLACES** HP10 in the cBot Cluster Generation Reagent Plate) is possible though. **ATTENTION:** Do not add the **CSP** ● Version 2 to the HP10 solution! A primer mixture would result in low clusters calls and the resulting reads would be contaminated by poly(T) stretches. Always use fresh HT1 and add the **CSP** ● Version 2 / HT1 dilution to the empty and rinsed well.

14. Appendix G: 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.

In QuantSeq REV (Cat. No. 016), the Read 1 linker sequence is located at the 5′ end of the oligodT primer. For Read 1, a Custom Sequencing Primer (included in the kit) has to be used. With QuantSeq REV it is possible to exactly pinpoint the 3′ end during Read 1. The reads generated here during Read 1 reflect the cDNA sequence, so they are in a strand orientation opposite to the genomic reference. For paired-end sequencing we strongly recommend using QuantSeq REV (Cat. No. 016).

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.

De-Multiplexing

External Barcodes: The barcode is contained in the Index Read, and demultiplexing can be carried out by the standard Illumina pipeline.

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. 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 be removed from the set.

While single-read sequencing does not require any trimming using QuantSeq REV (Cat. No. 016), paired-end sequencing may require the first 12 nucleotides of Read 2 to be trimmed. Alternatively, also here the STAR Aligner could be used with the number of allowed mismatches being set to 16 for paired-end reads.

Alignment

At this point the filtered and trimmed reads can be aligned with a short read aligner to the reference genome.

STAR aligner or TopHat2 can be used for mapping **QuantSeq FWD** (Cat.No. 015) data. The reads may not land in the last exon and span a junction. In case of no detected junction, TopHat2 may run into difficulties.

For **QuantSeq REV** (Cat.No. 016) we do not recommend using TopHat2, since there is hardly a need to search for junctions. Nearly all sequences will originate from the last exon and the 3'untranslated region (UTR). Hence, Bowtie2 or BWA can be used for mapping in this case.

Annotation

Mapping only the 3' end of transcripts requires an annotation which covers the 3' untranslated region (UTR) accurately. Conservative annotations might decrease the power of correct gene quantification after mapping, especially in case of QuantSeq REV (Cat. No. 016). 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.

Please visit our website (www.lexogen.com) for an up-to-date table of suggested species-specific annotations and comments.

More information about the principal data analysis can be found under www.lexogen.com.

15. Appendix H: References

Zimmermann, J et al., Automated Library Preparation using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (E7370) on the HAMILTON® STAR™ Line, Application Note, New England Biolabs, Inc. 2014.

16. Appendix I: Revision History

Publication No.	Change	Page
015UG065V0100	Initial Release autoQuantSeq 3' mRNA-Seq for Hamilton STAR/STARlet	
015UG065V0101	Reverse transcription: Incubation temperature changed to 42 °C Additional manual intervention and thermocycler program New labware option (plate) added Updated handling recommendation	6 6, 19 13 15

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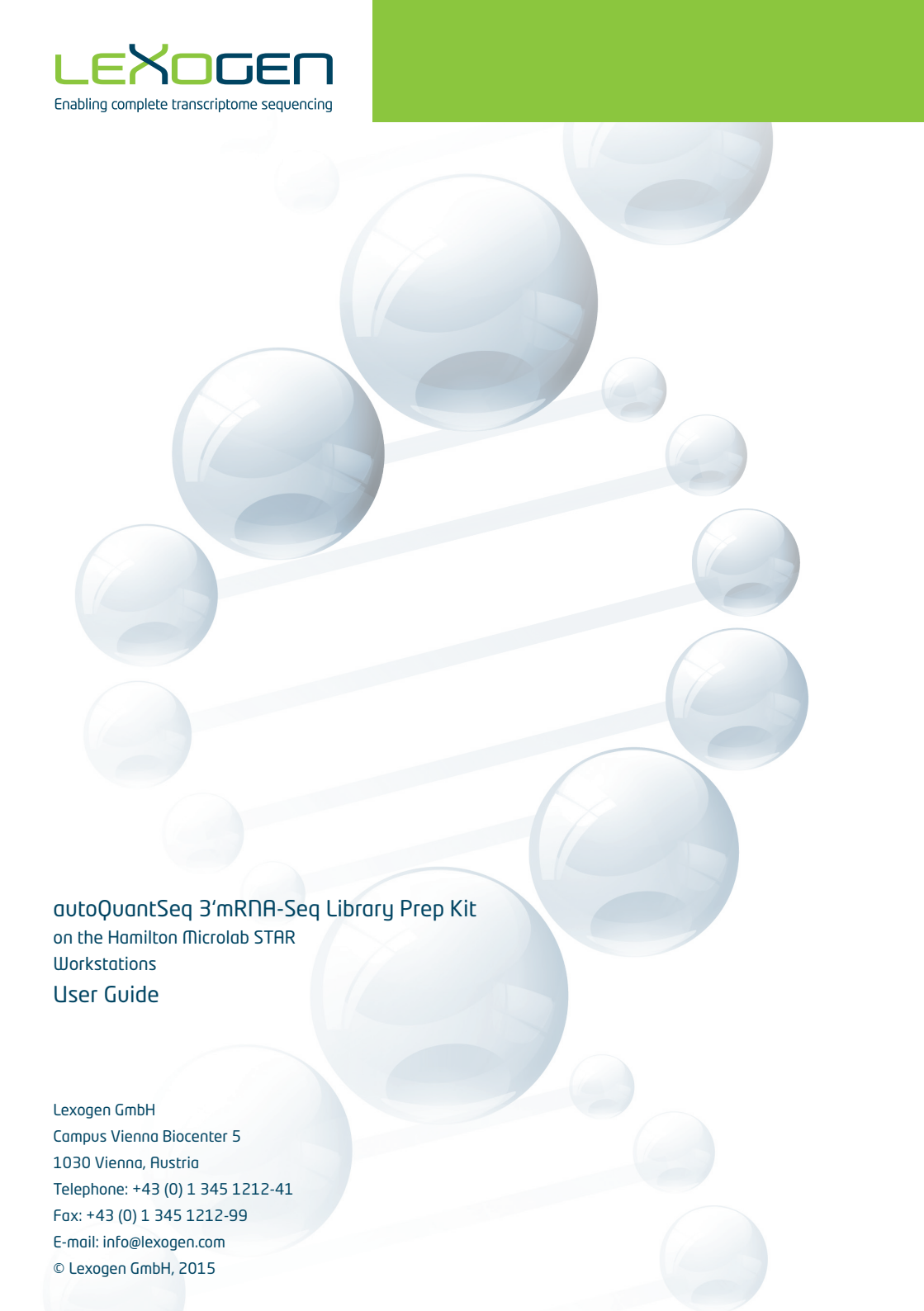

autoQuantSeq 3'mRNA-Seq Library Prep Kit

on the Hamilton Microlab STAR Workstations

User Guide

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