LEXOGEN

Enabling complete transcriptome sequencing

AUTO QUANT™ SEQ3

Simply counting 3' ends

3'mRNA-Seq Library Prep Kit
on the PerkinElmer Sciclone/Zephyr NGS 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 96 Illumina-compatible libraries from polyadenylated RNA within 7 hours. It runs on the Perkin-Elmer Sciclone NGS and Zephyr NGS 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 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.

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.28).

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.37, for a schematic representation of the finished library). Library quantification can be performed with standard protocols and is further discussed in Appendix D (p.34). Libraries are compatible with single-end or paired-end sequencing. Barcodes can be introduced during the PCR amplification step as

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

standard external barcodes (Appendix E, p.36). 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.41).

Automating the process of library preparation has the advantage of avoiding sample tracking errors while dramatically increasing throughput. autoQuantSeq is running on the liquid handler platforms by PerkinElmer. In the protocol described in this user guide, the prePCR phase is programmed for the Sciclone NGS Workstation whereas the postPCR phase (cleanup) is programmed for the Zephyr NGS Workstation. However, the postPCR phase can also be implemented on a Sciclone NGS workstation (ask Lexogen for details). Running prePCR and postPCR processing on separate machines, potentially in separate rooms, substantially reduces the risk of cross-contamination of the prePCR samples by PCR products.

autoQuantSeq is typically used for preparing 96 barcoded libraries. The set of 96 barcodes is included in the QuantSeq kit on a 96-well Barcode Plate. The liquid handler program allows for processing of samples in multiples of 8 preps (full columns of a 96-well plate) up to and including 96. If fewer than 96 samples are to be processed, the barcodes can be selectively transferred from the Kit Barcode Plate to a user-specific 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 placing the plates 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. Two different workflow options are available at the beginning of the protocol. The microplates can either be all filled directly by the operator before the run (which requires more manual preparation work but less machine time), or some of the reagents can be broadcasted to the microplates from a master deepwell (less manual work, longer machine run).

Thermal treatment can be done off-deck (using an external thermocycler, recommended) or on-deck (using built-in thermolocators).

The whole autoQuantSeq protocol (96 samples, with automated plate filling) can be run in 7 hours in total, including about 50 minutes of manual setup time. Since the individual protocol phases run on separate machines, further throughput enhancement can be achieved by parallelizing the workflow.

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 on the Sciclone NGS Workstation

Automated Plate Filling

In this optional step some of the reagents are broadcasted from a Master Plate to the microplates to save manual preparation time.

If Master Plate is used (this option can be set in the workbook), the operator has to prepare the run by filling the Master Plate itself, the Ethanol Plate, and the Sample Plate. The Barcode Plate comes prefilled in the QuantSeq kit. The protocol starts with transferring reagents from stock wells of the deepwell Master Plate to individual wells of the rest of the microplates. In the Master Plate, the reagents are either filled in a single deepwell or in a column of 8 wells (if the total stock volume needed would exceed the capacity of a single deepwell). Within this step, two microplates for the Phase 3 on the Zephyr are also filled. The operator is then asked to remove them from the Sciclone deck and store them at +4 °C until they will be needed.

If Master Plate is not used, the operator has to fill all the microplates with all the reagents according to the instructions in the workbook. This may be an option for processing substantially less than all 12 sample columns.

The plates containing RNA samples, enzymes, and some other reagents are now placed on thermolocators held at +10 °C.

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 First Strand cDNA Synthesis Mix 1 (**FS1**, 5 µl; all volumes specified are per prep) and the master mix **FS2 / E1** (prepared manually from the First Strand cDNA Synthesis Mix 2, **FS2**, 9.5 µl, and the Enzyme Mix 1, **E1**, 0.5 µl) are pre-arrayed to a clean 384-well plate. The reagent **FS1** (5 µl) is then transferred simultaneously from the 384-well plate 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. 33): If on-deck thermal treatment is used (this option can be set during installation), the machine stops and asks operator to seal the sample plate with a film, and resume the run. The sample plate is then moved to a thermolocator preheated to 85 °C, incubates there for 3 minutes, and is moved to another thermolocator preheated to 42 °C. The

machine stops and asks operator to unseal the plate and resume the run. If off-deck thermal treatment is used, the incubation at 85 °C and cooling down to 42 °C has to be done by the operator on an external thermocycler. The thermally treated plate is to be placed on deck unsealed and the run is to be resumed.

The **FS2 / E1** reagent (10 µl) is transferred simultaneously from the 384-well plate to the wells with samples and mixed. The plate is incubated for 15 minutes at 42 °C. If off-deck thermal treatment is used, this incubation has to be done by the operator on an external thermocycler. The thermally treated plate is to be placed on deck unsealed and the run is to be resumed. During this incubation, the RNA Removal Solution 1 (**RS1**, 5 µl) and the RNA Removal Solution 2 (**RS2**, 5 µl) are pre-arrayed to the 384-well plate.

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 simultaneously from the 384-well plate to the wells with samples and mixed.

If on-deck thermal treatment is used, the machine stops and asks operator the seal the sample plate with a film, and resume the run. The sample plate is then moved to a thermolocator preheated to 95 °C, incubates there for 10 minutes, and is moved to another thermolocator preheated to 25 °C. The machine stops and asks operator to unseal the plate and resume the run. If off-deck thermal treatment is used, the incubation at 95 °C and cooling down to 25 °C has to be done by the operator on an external thermocycler. The thermally treated plate is to be placed on deck unsealed and the run is to be resumed.

The **RS2** reagent (5 µl) is transferred simultaneously from the 384-well plate 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.

If on-deck thermal treatment is used, the machine stops and asks operator to seal the sample plate with a film, and resume the run. The sample plate is then moved to a thermolocator preheated to 98 °C, incubates there for 1 minute, and slowly cools down to 25 °C. (This ramp takes approximately 15 minutes, corresponding to less than 0.1 °C/second). The plate is then moved to another thermolocator preheated to 25 °C. The machine stops and asks operator to unseal the plate and resume the run. If off-deck thermal treatment is used, the incubation at 98 °C and cooling down to 25 °C has to be done by the operator on an external thermocycler. The thermally treated plate is to be placed on deck unsealed and the run is to be resumed.

The plate is incubated at 25 °C for 30 minutes. During this incubation, the **SS2 / E2** reagent (prepared manually from the Second Strand Synthesis Mix 2, **SS2**, 4 µl, and the Enzyme Mix 2, **E2**, 1µl) and the **PCR / E3** reagent (prepared manually from the PCR Mix, **PCR**, 7 µl, and the Enzyme Mix 3, **E3**, 1µl) are pre-arrayed to a new clean 384-well plate.

The **SS2 / E2** reagent (5 µl) is transferred simultaneously from the 384-well plate 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 dispensed to the sample wells, mixed, and incubated on a thermoshaker at 20 °C for 5 minutes at 500 rpm agitation. The plate is then placed on the magnet, the beads are separated, and the supernatant (80 µl) is removed. The beads are resuspended in Elution Buffer (**EB**, 40 µl), mixed well and incubated on a thermoshaker at 20 °C for 2 minutes at 500 rpm agitation. Purification Solution (**PS**, 72 µl) is added to the sample, mixed, and incubated again at 20 °C for 5 minutes at 500 rpm agitation. The plate is placed on the magnet, the beads are separated, and the supernatant (112 µl) 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, 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 20 °C for 2 minutes at 500 rpm agitation. The plate is placed on magnet, the beads are separated and the eluate (17 µl) is transferred to a clean plate. The PCR mix with enzyme (**PCR / E3**, 8 µl) is transferred simultaneously from the 384-well plate to each well and mixed thoroughly. The Barcodes (**BC**, 5 µl) from the Barcode Plate are also added simultaneously and mixed. The Sample Plate is then moved to the thermolocator where it is held at +10 °C. The pipetting head of the robot is moved away to make the resulting plate 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). Preferably, Phase 2 and Phase 3 (see below) of the protocol are run in separated rooms to avoid any possible contamination of pre-PCR samples.

Phase 3–PostPCR on the Zephyr NGS Workstation

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

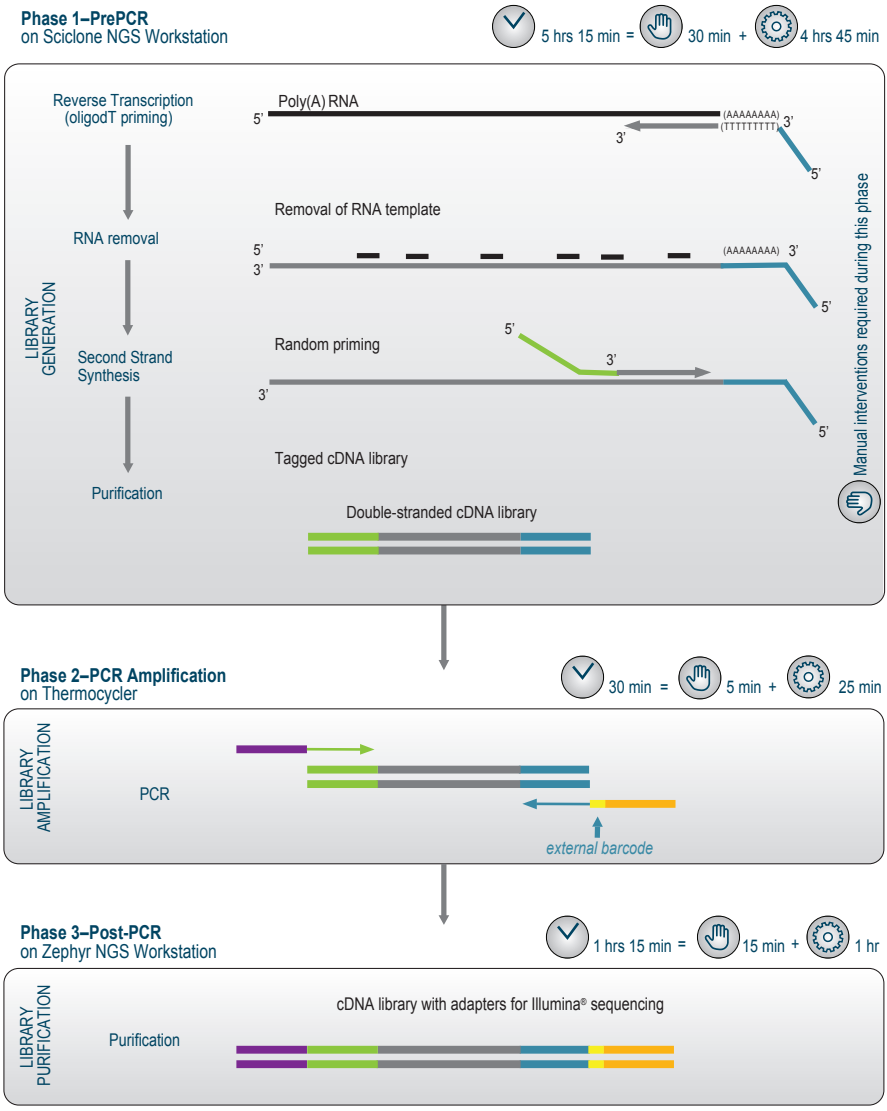


Figure 1. Schematic overview of the QuantSeq FWD library preparation workflow (Cat. No. 015). For QuantSeq REV (Cat. No. 016) the position of adapters for Read 1 (green) and Read 2 (blue) are switched. The execution times are given for 96 preps, with Master Plate used.

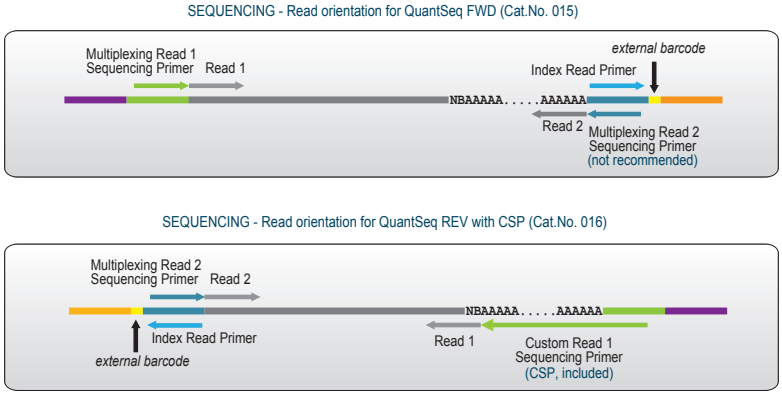


Figure 2. Sequencing Read orientation for QuantSeq FWD and QuantSeq REV. For QuantSeq FWD, Read 1 reflects the mRNA sequence. Paired-end Sequencing is not recommended for QuantSeq FWD. QuantSeq REV is suitable for paired-end sequencing, and Read 1 reflects the cDNA sequence. A Custom Sequencing Primer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

For this phase, the standard PostPCR SPRI Cleanup Application for the Zephyr NGS Workstation is used. The PCR product (27 µl from each well) is purified using 27 µl of Purification Beads reagent (**PB**, 1x reaction volume), washed twice with Ethanol, and then eluted in 20 µl of **EB**.

The samples are dispensed into the Purification Beads plate.

At this stage, the user is prompted to remove the original sample plate from the deck. This is the only manual intervention required during the run in Phase 3.

The samples and the Purification Beads (**PB**) are mixed and transferred to a clean plate. The plate is incubated for 10 minutes at 25 °C with 500 rpm agitation on a thermoshaker, then moved to the magnet, and incubated for another 10 minutes. The supernatant is removed and discarded.

An Ethanol (80%) wash step (150 µl per well) with 30 second incubation time is performed twice. The remaining Ethanol is removed from the sample plate, and the plate is moved to the shaker where the beads can dry for 3 minutes.

The Elution Buffer (**EB**, 20 µl) is transferred to the Sample Plate, mixed, incubated for 3 minutes, and mixed again. The Sample Plate is then placed to the magnet, the beads are separated for 3 minutes, and the eluate without beads is transferred to a clean plate. Phase 3–PostPCR is finished, and the user is prompted to remove the resulting plate from the deck.

After this step the amplified and purified libraries (20 µl in each well) are available in the resulting plate.

The autoQuantSeq protocol is finished.

3. Kit Components and Storage Conditions

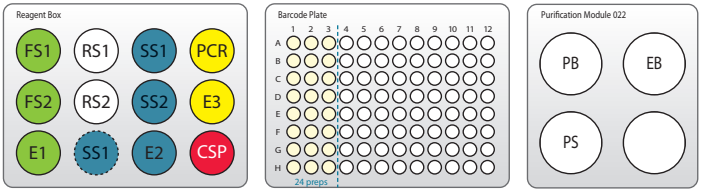


Figure 3. Location of kit components. For the 24 prep kit the dotted SS1 tube is missing and the barcode plate is only filled with set 1-3 (up to the blue dotted line). CSP is only required and included for QuantSeq REV (Cat. No. 016.24, Cat. No. 016.96, Cat. No. 016.2x96).

Kit Component	Tube Label	Volume* needed for		Storage
		24 preps (Master Plate not used)	96 preps (Master Plate used)	
First Strand cDNA Synthesis Mix 1	**FS1** ●	168.0 µl	651.0 µl	-20 °C
First Strand cDNA Synthesis Mix 2	**FS2** ●	279.3 µl	1109.6 µl	-20 °C
Enzyme Mix 1	**E1** ●	14.7 µl	58.4 µl	-20 °C
RNA Removal Solution 1	**RS1** ●	168.0 µl	651.0 µl	-20 °C
RNA Removal Solution 2	**RS2** ●	168.0 µl	651.0 µl	-20 °C
Second Strand Synthesis Mix 1	**SS1** ●	404.0 µl	1743.0 µl	-20 °C
Second Strand Synthesis Mix 2	**SS2** ●	134.4 µl	520.8 µl	-20 °C
Enzyme Mix 2	**E2** ●	33.6 µl	130.2 µl	-20 °C
PCR Mix	**PCR** ●	213.5 µl	894.3 µl	-20 °C
Enzyme Mix 3	**E3** ●	30.5 µl	127.8 µl	-20 °C
Barcode Plate (96-well plate)	**BC**	8.5 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**	1341.0 µl	5362.0 µl	+4 °C
Purification Solution	**PS**	2067.0 µl	8266.0 µl	+4 °C
Elution Buffer	**EB**	2445.0 µl	10080 µl	+4 °C

** only required for QuantSeq REV 016

*including all technical and manipulation surpluses

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.

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.37. Also forward this information to your sequencing facility before starting a sequencing run.

4. User-supplied Consumables and Equipment

Reagents

Reagent	Supplier
80% fresh EtOH (about 80 ml for 96 preps)	Various

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

Sciclone NGS / Zephyr NGS Workstation Accessories

Accessory	PerkinElmer Part No.
Agencourt® 96-ring magnet (1 for Sciclone, 1 for Zephyr)	CLS 128316
Inheco 96-well adapters (2 for Sciclone, 1 for Zephyr)	CLS 128372
Inheco 96-well adapter/shaker (1 for Sciclone, 1 for Zephyr)	CLS 100852
Inheco 384-well adapter (1 for Sciclone)	CLS 100853

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.28) for more information on RNA quality. Consult Appendix D (p.34) for information on library quantification methods.

Sciclone NGS / Zephyr NGS Software Requirements

Maestro software 6.0 patch 44 or newer must be installed on the control PCs for both Sciclone NGS and Zephyr NGS.

On-deck Consumables

Consumable	Description	PerkinElmer Part No.	Original Vendor and Part No.	No. Used per Run (96 samples, Master Plate used)
PCR plates	Bio-Rad 96-well full skirt hard-shell plate, or 4titude Frame-Star 96-well skirted PCR plate	600 8870 N/A	Bio-Rad HSP-9631 4ti-0960	11
384-well plate	Microplate 384-well, round bottom	600 8890	Corning, Inc. 3672	2
Boxed tips	Pipette tip 150µl , filtered, sterile	111426	-	12
Deepwell plates	Deepwell-06 POS, square 2.0 ml well, poly-pro, Seahorse	600 8880	Seahorse Bioscience 201379-100	5
Lids	946 Lid-universal, robotic friendly, polystyrene	600 0030	Seahorse Bioscience 200856-100	3

5. General Guidelines

Liquid Handler Usage

- Always wear gloves when touching the liquid handler casing, the deck, the Inheco controllers, 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.
- Before each run, lubricate the O-Rings of the Disposable Tip Array with the lubricator box. See Sciclone User manual or Zephyr User manual.
- At some points of the protocol used consumables such as lids or plates are discarded via the waste chute. Make sure a suitable basket or a box can hold the consumables falling out of the chute and prevent any potential damage to the surrounding equipment.
- 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 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 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 is similar to other protocols on the PerkinElmer liquid handlers but requires some experience and skills. Therefore, we recommend asking for Lexogen support. The following description is just for your information.*

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 which may supersede the instructions given in this manual. Unpack the archive and copy the files to the following destination folders on your PC connected to the Sciclone:

Folder LEXOGEN with all subfolders to
C:\ProgramData\CaliperLS\Maestro

File LEX_autoQUANTSEQ_prePCR_YYYY-MM-DDn.zip to
C:\ProgramData\CaliperLS\Maestro\Exported Archives

If you are updating an existing installation, we recommend to rename the old files and folders first, then load the new versions. You can keep the old files for reference.

First Start

Start the Maestro software, open a new application (**File > New Application**) and import the archive file you have just downloaded (**File > Import > Import Archive**). You find the file in C:\ProgramData\CaliperLS\Maestro\Exported Archives. In the **Import Archive Options** dialog, keep the **Current Application** and **VSTA Macros** checkboxes checked and click **OK**. You will be immediately prompted to save the application in the **Save Maestro Application** file dialog. Save the application under the suggested name.

Fine Tuning Application

The application has all critical pipetting heights identified and appropriate variables created to control those pipetting heights. The actual values of these variables used to position tips precisely at the bottom of consumable wells are instrument and application specific. Setup (teaching) must be done on the instrument which will be used for real runs.

The variables to be taught are summarized in the UTIL_SetGlobalOffsets method of the application. They are named *g_Offset_NNN* and *L_correction_N_column*. The teaching itself consists

of manual control of the gantry to drive the head down until the tips touch the bottom of the wells in the respective consumable dedicated for the given location on deck. Each variable has a description with specification of its teaching conditions (location on the deck and type of consumable used at this location). The optimum values found during the manual teaching are then saved to the code of the UTIL_SetGlobalOffsets method.

When updating the application to a new version, the currently validated values of the *g_Offset_NNN* and *L_correction_N_column* have to be copied to the new application.

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. The wet test can be done using water in place of wash solutions, 10 % PEG or 10 % glycerol in place of enzyme solutions, Ethanol, and some Purification Beads (not necessarily in all rows), if available. Alternatively, ask Lexogen for a kit with dummy liquids for wet testing (Cat. No. 019.24). The wet test will ensure the liquid handling is clean and the beads are not lost during cleanups.

7. Detailed Protocol

Read all the instructions carefully prior to beginning the run. This description assumes the Application Phase 1 is already installed on the control PC of the Sciclone NGS, and the Application Phase 3 is already installed on the control PC of the Zephyr NGS.

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.
- If necessary, boot up the system by first starting the Sciclone and the Inheco units, then starting the Sciclone control PC. Also start the Zephyr and its Inheco units, then start the Zephyr control PC.

7.2 Workbook Preparation

Open the Microsoft Excel workbook in this filepath of the Sciclone control PC:
C:\ProgramData\CaliperLS\Maestro\LEXOGEN\Workbooks\autoQUANTSEQ Library Prep Workbook.xls

In the worksheets *Reagent Plates PrePCR(Sciclone)* and *Reagent Plates PostPCR(Zephyr)* of this workbook (Fig. 4), you will find pipetting schemes as well as reagent preparation guidelines for the respective protocol phases. Fill the yellow marked cells in the header of the *Reagent Plates PrePCR(Sciclone)* worksheet (assay ID, date, number of samples, Master Plate usage, and the Denaturation Step options). 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.

For the **Number of samples**, you can select any multiple of 8, from 8 to 96 (the application processes full columns of 8 samples each). The standard setting is 96 samples. If you intend to process less samples (e.g., for testing), refer to the following note.

Processing less than 96 samples

The application which is setup to use the first N sample columns only, will always take the barcodes from the columns 1, 2, … , N of the Barcode Plate. In the Kit Barcode Plate, however, the first columns might have been used in the previous run. Therefore, the barcodes have to be manually transferred from the Kit Barcode Plate to the wells in the first N columns of the Barcode Plate placed on the deck prior to application start. See Chapter 7.3 Plate preparation / P5 Plate – Barcodes.

Master Plate Usage Option

In the **Use Master Plate:** cell, select between **YES** (manual plate preparation time is reduced, machine run time is longer), or **NO** (more manual preparation work, shorter machine run time).

Master Plate used (option YES)

This option is recommended for high-throughput processing, typically for processing the whole plate (96 preps). The operator has to prepare the run by filling the Master Plate itself, the Ethanol plates, and the Sample Plate. The Barcode Plate comes prefilled in the QuantSeq kit. The protocol starts with transferring reagents from stock wells of the deepwell Master Plate to individual wells of the rest of the microplates. In the Master Plate, the reagents are either filled in a single deepwell or in a column of 8 wells (if the total stock volume needed would exceed the capacity of a single deepwell). Within this step, two microplates for the Phase 3 on the Zephyr are also filled. The operator is then asked to remove them from the Sciclone deck and store them at +4 °C until they will be needed.

Refer to p.24 below for detailed instruction on filling the Master Plate.

Master Plate not used (option NO)

We recommend to use this option for processing smaller amounts of samples (substantially less than all 12 sample columns). In this case the operator has to fill all the microplates with all the reagents according to the instructions in the workbook.

For comparison, the protocol parameters for processing 96 samples (1 full plate) are summarized in the following table:

96 samples, Phase 1–PrePCR only	Master Plate used (option YES)	Master Plate not used (option NO)
Manual preparation time [minutes]	30	60
Machine run time [minutes]	285	210
Tip consumption [number of tip boxes]	8	8

Refer to table on p.21 for an overview of which plates have to be filled manually for a particular Master Plate usage setting.

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 FFPE samples and denaturated samples, see Appendix C, p.33).

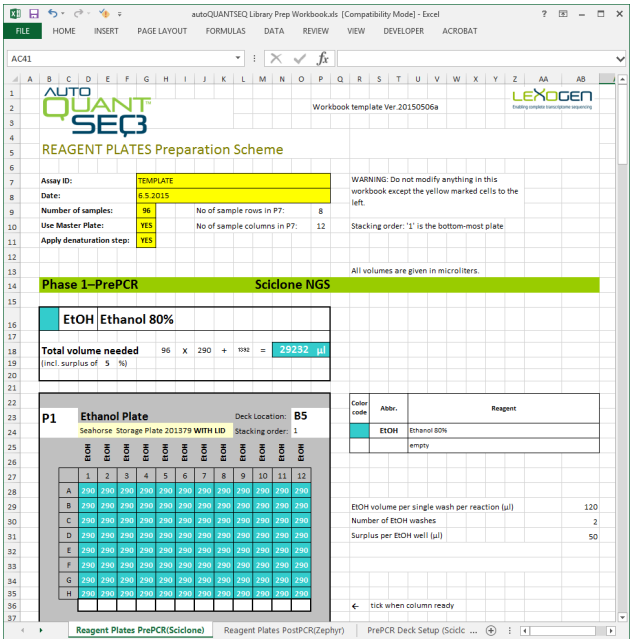


Figure 4: A fragment of the autoQuantSeq workbook. Note that the actual layout of the spreadsheet in the software distribution may differ slightly from the layout shown.

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.

7.3 Plate Preparation

For reference, the plates used in the protocol are named P1, P2, etc. The numbering of the plates corresponds to the recommended preparation sequence. Plates P21, P22, and P23 are required for the Phase 3. Manual plate filling can be partially substituted by automated filling from the M1 Master Plate (if the Master Plate used option is set to YES). An overview of which plates have to be filled manually in a particular case is shown in the table below.

For processing 96 samples, the plate P5 is the QuantSeq Kit Barcode Plate. For smaller number of samples, the P5 plate may have to be prepared by the operator. For the deck setup, you will also need some empty plates. Some of them will be used for reagent transfer during the protocol, and must be clean and sterile. Some other plates are used as spacers, can be non-sterile and may be reused.

Plates to be filled manually	
if Master Plate is used (option YES)	**if Master Plate is not used** (option NO)
P1 (Ethanol) **P5** (Barcodes; typically, this is the kit barcode plate) **P7** (Samples) **M1** (Master Plate) **P21** (Ethanol)	**P1** (Ethanol) **P2** (Purification Beads) **P3** (Purification Solution) **P4** (Elution Buffer) **P5** (Barcodes; typically, this is the kit barcode plate) **P6** (Reagents) **P7** (Samples) **P21** (Ethanol) **P22** (Purification Beads) **P23** (Elution Buffer)

Understanding the Plate Preparation Worksheets

The detailed plate setup is described in the Microsoft Excel workbook. The worksheets *Reagent Plates PrePCR (Sciclone)* and *Reagent Plates PostPCR (Zephyr)* also contain recipes for master mixes used in the run.

Refer to Figure 5 for the explanation of the worksheets. We recommend to follow the worksheet from top to bottom and to prepare the plates one after another in the given order. For each plate, check whether the reagents to be filled in this plate are readily available in the kit, or a master mix (or more master mixes) has/have to be prepared first. Prepare all master mixes first, then proceed with transferring the reagents into the plate wells, column after column. Cover the plate with a lid if this is indicated.

Specific instructions for selected individual plates are given below.

P5 Plate – Barcodes

In the QuantSeq kit, the Barcode Plate comes prefilled with the barcoded primers and sealed with cap stripes. The plate can be either used on the deck (for a full 96 sample run using all barcodes), or the selected barcodes can be transferred to a new plate.

- The Kit Barcode Plate has to be stored at -20 °C.
- Before the run, remove the lid (remove the sticky tapes holding the lid but do not remove the cap stripes), thaw the plate for 5 minutes in a thermocycler at 25 °C. Visually inspect the wells to ensure that they all are completely thawed.
- Centrifuge the plate shortly to collect all of the barcode at the bottom of the well.
- Hold the plate tightly and press firmly against the lab bench. Slowly and gently remove the cap stripes. Take care not to spray any liquid from the well around as this would cause crosscontamination.
- Cover the plate with the lid again. Make sure the lid and the plate are free from any rests of the sticky tape, in particular at the side walls. The plate is now ready to be placed on deck as the P5 plate.

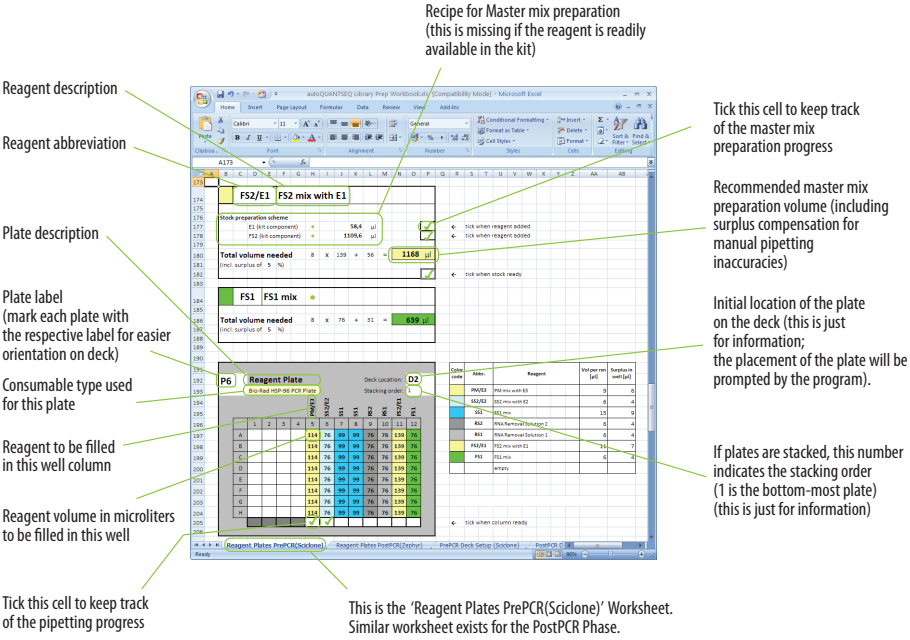


Figure 5: Understanding Worksheet.

If **processing less than 96 samples** (i.e., you do not intend to use up all barcode primers in this run), proceed as follows:

- Remove just the cap stripes from the wells you want to use in this run.
- Using a pipette or an 8-tip multichannel pipettor, transfer the full volume of the barcodes in the selected wells of the Kit Barcode Plate to a new Bio-Rad HSP-96 plate (the new P5 plate). Fill the new P5 plate column-by-column, starting from column 1. Cover the new P5 plate with a new lid after filling. The new P5 plate is now ready to be placed on deck.
- Put the plastic cover that came with the Kit Barcode Plate back on the plate. Before doing so, you may want to cover some of the empty wells with a cap stripe just to ensure the plastic cover sits better in place.
- Refreeze the Kit Barcode Plate and store at -20 °C.
- It is recommended to use the Kit Barcode Plate for a maximum of four thaw/refreeze cycles.
- The filling scheme of the Kit Barcode Plate with barcode sequences can be found on p.36.

P6 Plate – Reagents

You have to fill this plate only if you selected not to use the Master Plate.

Thaw the QuantSeq kit reagents stored at -20 °C on ice. Ensure that each supplied reagent has been fully thawed, gently mixed, and spun down before use. After adding the appropriate volumes of reagents in nuclease-free tubes, gently mix, and spin again to collect all liquid at the bottoms of the tubes. Keep the master mixes on ice and promptly return unused portions of reagents to -20 °C storage.

Pipet carefully into the bottom of the wells and avoid trapping air or creating bubbles. If necessary, spin the plate briefly in a plate centrifuge to ensure all reagents are at the bottom of the wells.

Store the plate on ice or at +4 °C until you place it on deck.

P7 Plate (Samples)

Prepare this plate as the last one in the preparation process, immediately before starting the run. Observe the general recommendations for RNA sample handling given on p.14.

Cover the plate with a lid after filling and store on ice. Place the plate on the deck when prompted by the application. This will ensure the thermolocator at the respective position is already pre-cooled to the plate storage temperature.

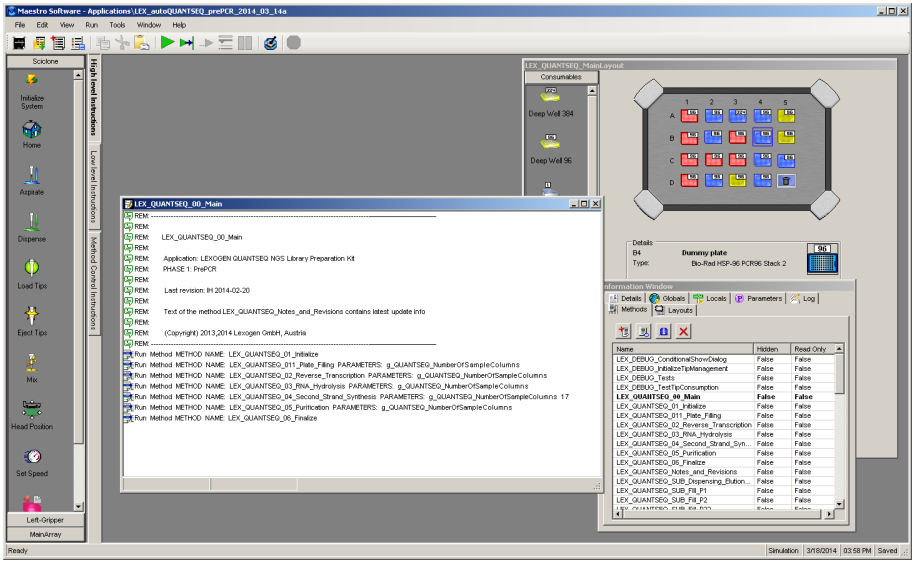


Figure 6: Maestro window with the autoQuantSeq application loaded.

M1 Master Plate

You have to fill this plate only if you selected to use the Master Plate. Most reagents are filled in a single deepwell of the plate but some have to be distributed into several wells due to extensive volume. Make sure you pipet the reagents to the bottom of the wells.

7.4 Starting the Application on Sciclone NGS

Start the Maestro software on the Sciclone control PC. Load the autoQUANTSEQ application by selecting **File > Open Application**. In the **Open Maestro Application** dialog, select the LEX_autoQUANTSEQ_prePCR application (make sure you select the correct one if you have stored more versions) and click **Open**. If the application is loaded properly, a window with the LEX_QUANTSEQ_00_Main method will be shown at the top (Figure 6).

You do not need to setup the deck prior to starting the application. Instead, wait for the application to prompt you to do so.

Start the application main method. An introductory application window will appear. Click the green **Continue** button. Throughout the application, green controls indicate the suggested (or typical) way of proceeding. The application window will always show the actual processing phase.

After a while, the application will show the parameters from the workbook. Check that the values in the workbook have been read correctly (for a quick check, make sure the Assay ID shown corresponds to your entry in the workbook). The most frequent cause of a problem (the application stops with an error message) is that the workbook is not at its correct location or is not readable.

If the workbook has been read correctly, press **Continue**.

7.5 Thermocycler programming

If the thermal treatment during Phase1 is to be done off-deck (this option has to be set in the code; ask Lexogen for details), we recommend to set up the programs for the thermocycler in advance. Four different thermal procedures are required during the run (see Chapter 7.7 below). 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 for 3 minutes • 42 °C hold Volume 10 µl, lid temperature 105 °C
Incubation #1A	• 42 °C for 15 minutes • 42 °C hold Volume 20 µl, lid temperature 105 °C
Incubation #2	• 95 °C for 10 minutes • 25 °C hold 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 hold Volume 45 µl, lid temperature 105 °C

7.6 Sciclone NGS Deck Setup

The application will now show a **Deck Setup Visual Check Dialog**. Browse through all pictures and hints presented. At this stage, place the consumables and the prefilled plates to their respective positions on deck as instructed by the pictures. The thermolocators holding the plates with sensitive reagents are now at the required storage temperature and the head of the liquid handler has moved to the back to free the manipulation space for the operator. Make sure your deck has been set up according to the instructions.

7.7 Phase 1–PrePCR Run

Pressing **Start run** in the Deck Setup Visual Check dialog will start the liquid and material handling process of the protocol. 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 will be given on the application panel. Follow them carefully. For thermal procedures, you may use the programs stored in the thermocycler (see Chapter 7.5 above). 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.

Phase 1–PrePCR which will take about 4 hours and 15 minutes (with Master Plate used). For the detailed step-by-step guide to the protocol, see the application workbook, worksheet *Detailed Protocol*.

7.8 After Phase 1–PrePCR Completion

When Phase 1–PrePCR is complete, the application will stop. Close the final dialog. The plate with the reagents prepared for the PCR amplification is located at the thermolocator T3 (location D2) and held at +10 °C.

Remove used tip boxes from the deck. Discard used plates. Empty the tip waste.

7.9 Phase 2–PCR Amplification

Take the plate from location D2, 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 program is

- 98 °C, 30 sec
- Repeat **12** times (refer to p.32 for more details on cycle number specification)
  - 98 °C, 10 sec
  - 65 °C, 20 sec
  - 72 °C, 30 sec
- 72 °C, 1 min
- 10 °C, ∞

Set the thermocycler for 30 µl of PCR mix. Use a heated lid (recommended temperature 105 °C) to prevent condensation. See Appendix B: PCR Cycles – Low Input Material (p.32) for information regarding optimization of library amplification. Start thermocycling.

We recommend to use the time for preparing the run on the Zephyr NGS (see Chapter 7.10).

7.10 Zephyr NGS Deck Setup

The Post-PCR SPRI cleanup is a separate Maestro application, running on the Zephyr NGS workstation (a different liquid handler for postPCR sample processing to avoid any possible cross-contamination of the pre-amplification samples).

Note: *Post-PCR SPRI cleanup applications are available for both Sciclone NGS and the Zephyr NGS workstations. The instructions here are for running the autoQuantSeq post-PCR SPRI application on the Zephyr NGS workstation. Ask Lexogen for a different setup.*

Fill the plates for Phase 3–PostPCR following the guidelines in the respective worksheet of the Microsoft Excel workbook. Make sure the Purification Beads are at room temperature and mixed well prior to pipetting. Place the plates on deck according to the workbook.

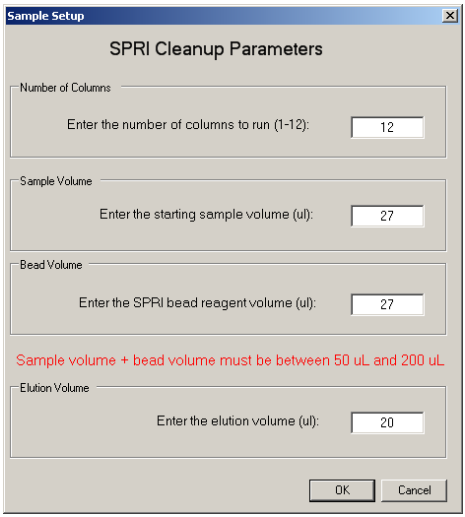


Figure 7: Introductory dialog of the Phase 3-PostPCR

7.11 Phase 3-PostPCR Run

Start the application main method. In the introductory dialog, fill the entries like follows (Figure 7): **Number of Columns** according to the actual setup; **Sample Volume** 27 µl; **Bead Volume** 27 µl; **Elution Volume** 20 µl.

You will be prompted to check the deck setup, and to start the liquid and material handling process. Shortly after start, you will be prompted to remove the original sample plate from the deck. This will be the last manual intervention required. The whole process will take about 55 minutes for a plate of 96 samples.

7.12 After Phase 3-PostPCR Completion

When the Phase 3–PostPCR is complete, the application will stop. The plate with the amplified and purified library is located at C2.

Remove used tip boxes from the deck. Discard used plates. 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.33.

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, funghi, 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.32).

With reduced total RNA input cycle numbers need to be adjusted accordingly (see Appendix B, p.32). 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 reactions (in the workbook, set the protocol for 8 samples, but only fill four of the wells, e.g., A1 to D1 with reagents), 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 of a 1:4,000 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 over-cycling 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.

Lower inputs (10 ng or less) require protocol adjustments. Contact Lexogen for details.

The table below comprises some results obtained from using different amounts of total RNA input using the manual preparation protocol. 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.28, 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	12
50 ng	72 µl	122	1000	318	196	93 %	67 %	23 %	3.1	17.0	15
10 ng	48 µl	122	1000	350	228	95 %	77 %	32 %	2.1	10.7	18
5 ng	48 µl	122	1000	292	170	94 %	66 %	17 %	2.1	12.1	20

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

ATTENTION: For input RNA amounts of 10 ng or less the amount of **PS** added was reduced (48 µl instead of 72 µl) to prevent sequencing through linkers and poly(T) stretches. Contact Lexogen for details how to adapt the automated protocol.

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, its 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 set up directly in the code. Contact Lexogen for details how to adapt the automated protocol.

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.28.

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 50ng (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.32). 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 two examples of expected NGS library parameters see Figures 8 and 9 which were amplified for 12 PCR cycles, eluted in 20 µl, and analyzed by a capillary electrophoresis instrument.

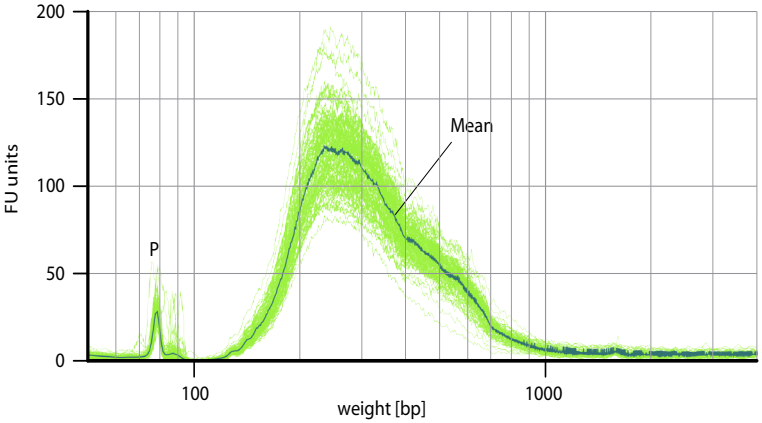


Figure 8: Electrophoretic traces of autoQuantSeq NGS libraries. 96 NGS libraries were generated and analyzed by a PerkinElmer LabChip GX electrophoresis instrument with a High Sensitivity DNA Kit. Identical starting material of 500 ng Agilent's Universal Human Reference RNA (UHR-RNA) each produced within 12 PCR cycles 20 µl libraries of 12.7±3.2 nM, or 253±65 fmol, with a total average length of 339.8±9.6 bp corresponding to 217.8 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.

157	104	99	127	120	125	73	110	126	104	81	98
157	149	140	139	117	158	165	137	135	116	94	111
94	77	99	80	100	90	74	82	82	91	82	60
74	60	93	79	108	79	82	99	134	98	68	65
84	99	107	121	93	105	114	92	103	104	109	92
70	114	146	97	157	91	92	74	91	118	90	73
98	96	102	68	94	85	121	59	105	97	77	87
90	97	99	98	118	69	96	78	111	92	72	64

Figure 9: Heat map showing relative yields compared to the average of 96 individually barcoded autoQuantSeq NGS libraries.

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.

QuantSeq is specifically designed for multiplexing 48 or 96 samples per sequencing lane (depending on the intended read depth).

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'

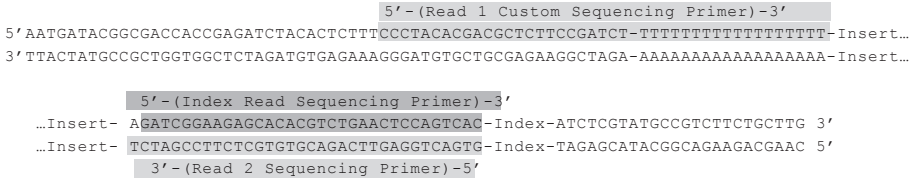
ATTENTION: We do not recommend paired-end sequencing for QuantSeq FWD (Cat.No. 015.24, Cat. No. 0.15.96, Cat. No. 0.15.2x96), as the quality of Read 2 would be very low due to the poly(T) stretch at the beginning of Read 2.

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: Glossary

Application

Under Application, we understand the software code and hardware setup for running a particular protocol on the liquid handler. This User Guide describes the autoQuantSeq application, i.e., the software and hardware components for automated library preparation using the reagents of the QuantSeq 3′ mRNA-Seq Library Prep Kit.

Barcode Plate

The Barcode Plate contains external barcodes (**BC**) allowing up to 96 samples to be sequenced per lane. In the QuantSeq kit, the barcodes come prefilled in an HSP-96 plate (8.5 µl per well). See Appendix E, p.36, for barcode sequence allocation in the plate. Typically, the Kit Barcode Plate can be readily used as the P5 plate on deck in the Phase1-PrePCR. If you plan to use the 96-prep kit for several machine runs you will need to transfer the reagents from the Kit Barcode Plate to a new HSP-96 plate.

Deck Setup

To prepare a machine run, consumables, some filled with reagents, have to be placed on deck. The correct allocation of the consumables on the machine deck is shown in the workbook, and detailed instructions for the placement are also given by the control program at start of each run. Particular deck setup depends on the number of preps in the given run, as well as on specific modifications of the protocol.

Master Plate

For automated plate filling, the reagents are manually filled into a 2 ml deepwell plate. Most of the reagents are filled in a single deepwell (the total volume required for all reactions), some of the reagents have to be filled in column of deepwells (due to the extensive total volume). The Master Plate is placed on deck and at the start of the protocol the liquid handler broadcasts the reagents from the Master Plate to the respective empty HSP-96 plates.

Plate Filling: Manual vs. Automated

Several HSP-96 microplates have to be filled with reagents prior to the run. To simplify the manual preparation, a major part of the pipetting work can be done by the liquid handler. In this case, the reagents are manually filled in a Master Plate (typically the whole reagent volume for the run in a single well). The filled Master Plate is placed on deck, and the HSP-96 plates are placed on deck initially empty. The reagents are automatically broadcasted to the HSP-96 plates. Manual work is herewith saved but the machine run takes longer. Manual or Automated plate filling can be selected in the workbook (option Use Master Plate:). Note that Ethanol (P1 plate), barcodes

(P5 plate), and RNA samples (P7 plate) have to be filled manually even if automated plate filling is activated. Automated plate filling can be activated for any number of preps from 16 to 96 but is only recommended for higher number of preps.

Pre-arraying

The pre-arraying maneuver is used in the protocol to make sure the reaction times are similar for all reaction wells of the plate. Instead of broadcasting a reagent (stored in a single column of a 96-well plate) sequentially to the individual columns of the plate where the reaction is already active, the reagent is sequentially (using 8 tips) broadcasted to an intermediate clean plate (aliquoted, typically, to all 96 wells). This broadcasting is called pre-arraying. At the given step of the protocol, the reagent is simultaneously (typically, using all 96 tips) transferred to the reaction plate. A 384-well plate is used as the intermediate plate, since there is room for four different pre-arrayed reagents per single well of a 96-well plate.

Thermal Treatment On-Deck vs. Off-Deck

The autoQuantSeq protocol is available with an optional modification for thermal treatment. The thermal steps (RNA denaturation, RNA removal, and second strand synthesis) can be performed on an external thermocycler ('off-deck'), or on the thermolocators in the liquid handler ('on-deck').

The on-deck alternative does not require any external equipment. At the given step of the protocol the machine stops and asks the operator for manual intervention. For on-deck thermal treatment, the operator has to take the plate from deck, seal it with film, spin shortly, place back on deck again, and resume run. The program will continue by moving the plate to a pre-heated thermolocator, running the given thermal treatment procedure, and moving the plate back. Then it stops again and asks the operator to take the plate, spin shortly, remove the seal, place back on deck, and resume the run.

For the off- deck alternative (recommended), the manual intervention includes taking the plate from deck, sealing with film, spinning shortly, placing on an external thermocycler, running the given thermal treatment procedure, spinning, unsealing, placing back on deck, and resuming run.

On-deck or off-deck thermal treatment can be selected in the code of the protocol at installation. Ask Lexogen for more details and/or support.

Thermolocator

The term Thermolocator is used to describe the positions of the liquid handler deck equipped with heater/cooler units, as well as the thermal devices themselves. The autoQuantSeq protocol assumes the deck of the Sciclone being equipped with four thermolocators, three of which (TL1, TL2, TL3, at locations A3, A4, and D2, respectively) are INHECO thermal units, and one (TL4, location D4) being an INHECO thermoshaker. The TL1 thermolocator is equipped with a 384-well adapter, the TL2, TL3, and TL4 are equipped with a 96-well adaptor each. The deck of the Zephyr

has to be equipped with two thermolocators, TL1 (with shaker) and TL2, at locations B3 and C2, respectively, both equipped with a 96-well adaptor. Note that the thermolocators themselves are hard-mounted, but the adapters (top plates for holding a 96-well plate, a 384-well plate, or similar) can be easily changed and their configuration may differ from other protocols run on the given liquid handler.

Workbook

The workbook is a Microsoft Excel file (currently, only Excel 97-2003 format is supported) containing data and useful information for the operator preparing a machine run of the protocol. In particular, the workbook contains detailed recipes for preparing master mixes of the reagents, filling schemes for the plates, deck setups, consumption summaries, detailed machine program of the application in human-readable form etc. Filling the workbook is the first step in preparing a run. In the workbook, the operator has to set up the protocol parameters for the run (such as number of samples, Using Master Plate option selection, etc.). All other data (such as reagents volumes, plate filling schemes etc.) are automatically recalculated. The workbook (or just selected worksheets) can then be printed out and used as a guide for manual plate preparation and as a guide throughout the execution. Moreover, the workbook file is directly read by the control program of the liquid handler, and the parameters of the run are set up accordingly. It is vital that the workbook file is not moved within the file system and/or renamed, as this causes error in application execution.

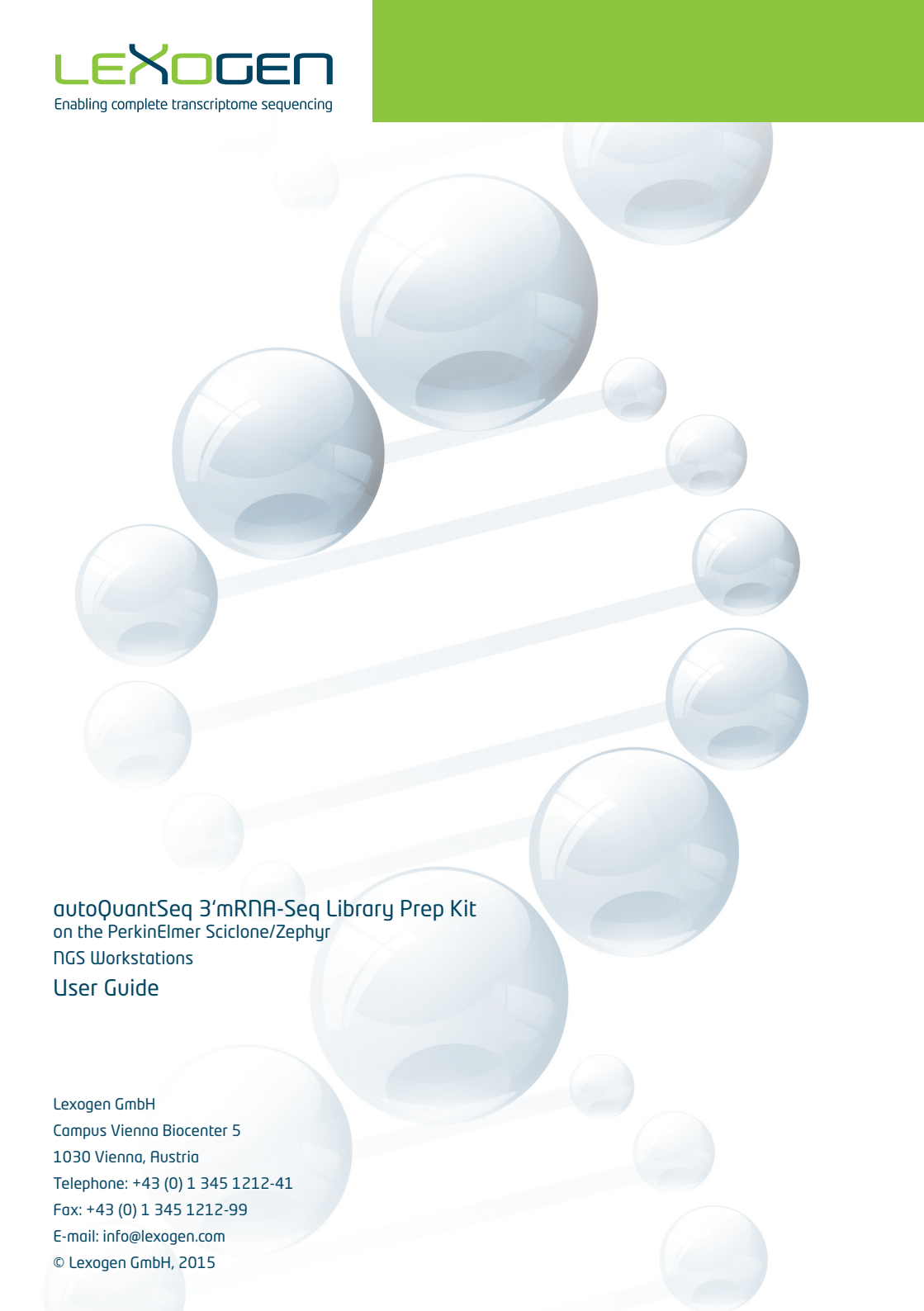
16. Appendix I: Revision History

Publication No.	Change	Page
015UG010V0111	Reverse Transcription incubation temperature changed to 42°C Additional manual intervention and thermocycler program New labware option (plate) added Updated handling recommendation	6, 7 25 13 15
015UG010V0110	General Update to match 015G009V0200	
May 10th 2014	Initial Release autoQuantSeq 3' mRNA-Seq	

Notes

LEXOGEN

Enabling complete transcriptome sequencing



autoQuantSeq 3'mRNA-Seq Library Prep Kit

on the PerkinElmer Sciclone/Zephyr

NGS Workstations

User Guide

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