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Automation of the Lexogen QuantSeq 3' mRNA Kit on the Agilent NGS Workstation Produces High-Quality Sequencing Libraries

Abstract

Lexogen QuantSeq 3' mRNA kits use oligo-dT primers to capture short fragments at the 3' end of poly-adenylated RNA, allowing for accurate expression profiling from various sample types, including formalin-fixed, paraffin-embedded (FFPE) tissue. The kits have options for low (0.1 to 10 ng total RNA) or standard (10 to 2,000 ng total RNA) input as well as dual or single indexing. QuantSeq was automated on the Agilent NGS Workstation. The various protocol options have been implemented in a single user interface for flexibility and ease of use. The NGS Workstation reduces hands-on time and maintains final library metrics such as concentration, mappability, and input-output correlation. Here, sequencing data generated from the automated protocol are analyzed. Universal Human Reference RNA (UHRR) with External RNA Control Consortium (ERCC) spike-ins was used to prepare eight libraries each of 100 ng and 250 ng input. The results show that libraries prepared on the NGS Workstation produce high-quality libraries with consistent size and concentration, high mappability, low percentage of ribosomal RNA (rRNA), and high input-output correlation, as calculated from ERCC spike-ins.

Introduction

QuantSeq is a straightforward method to prepare highly strand-specific mRNA libraries without the use of an additional poly-adenylated RNA enrichment or ribosomal depletion step. The kit comes in three types:

- FWD, generates reads toward the poly(A) tail that correspond to the mRNA sequence during Read 1 sequencing
- REV, generates reads corresponding to the cDNA sequence during Read 1 sequencing
- Flex, to sequence targeted regions using custom primers

Figure 1 displays and describes the QuantSeq method. This method produces one fragment per transcript, allowing for straightforward analysis because the number of reads mapped to a transcript is proportional to its expression. Samples can be highly multiplexed using the 384 available barcode combinations. The data analysis is simpler and more accurate than with standard RNA-Seq, as it is based on gene counting, and does not require FPKM calculations.

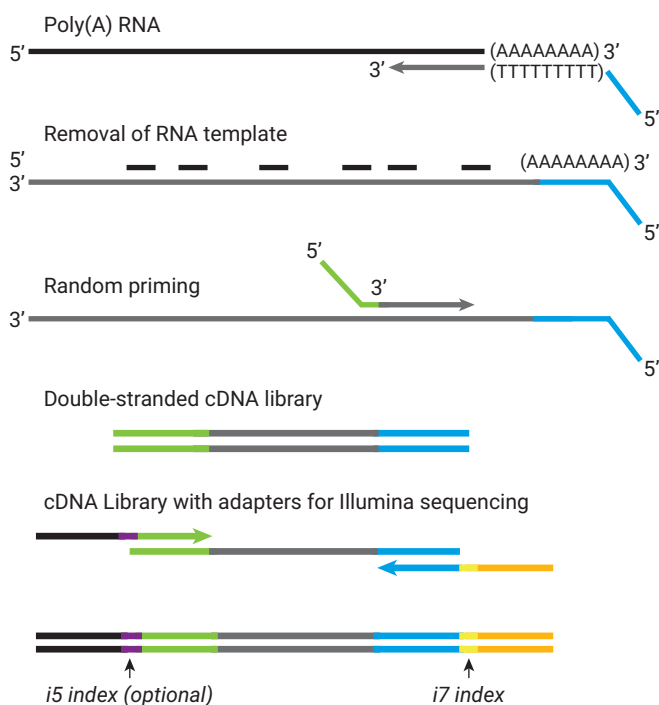


Figure 1. The QuantSeq 3' mRNA protocol produces mRNA libraries in 4.5 hours. Total RNA is reverse transcribed using oligo-dT primers. The RNA template is removed and the second strand is synthesized using random or targeted primers. The cDNA library is purified before being amplified with barcoded Illumina adapters. A final purification removes extra adapters that can interfere with sequencing.

Although the QuantSeq method requires minimal hands-on time, it can be reduced further by introducing automation. Additionally, automation reduces user error and increases throughput and reproducibility. The Agilent NGS Workstation is an automation platform featuring the Agilent Bravo Automated Liquid Handling Platform, Agilent BenchCel 4R Microplate Handler, and the Agilent Labware MiniHub (Figure 2). The Bravo has accessories for heating, cooling, shaking, and magnetic bead separation, allowing for most incubations to be completed on deck.

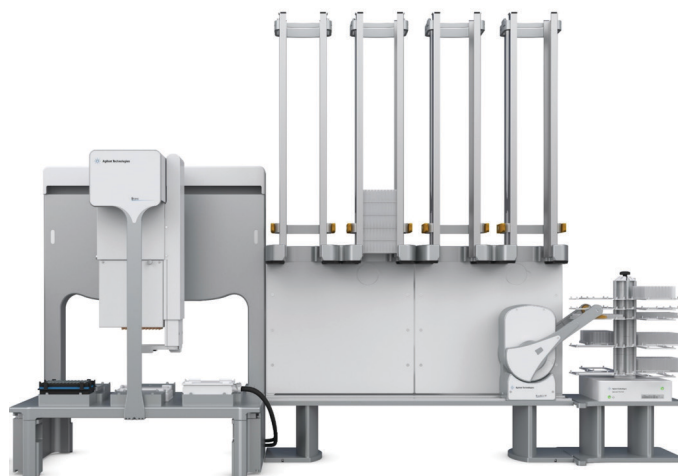


Figure 2. The Agilent NGS Workstation facilitates processing of up to 96 samples with maximum walkaway time. The NGS Workstation features the Agilent Bravo liquid handler (left), Agilent BenchCel 4R microplate handler with labware stackers (center), and MiniHub reagent carousel (right). The Bravo deck has nine deck positions, including two heating and cooling positions, one chiller position, one orbital shaker, and a removable 96-well magnet. The BenchCel brings tip boxes, labware, and reagents to and from the Bravo deck, facilitating maximum walkaway time.

The automation protocols were written in VWorks Automation Control software. A VWorks form interface was created to capture user input, such as protocol and reagent version, and display the Workstation setup (Figure 3). The form allows discrete, flexible protocols to be run from a familiar, easy-to-use interface. The protocol can accommodate simultaneous processing of 8, 16, 24, 36, 48, or 96 libraries per run.



LEXOGEN
QuantSeq RNA-Seq Kits

Parameters

- 1) Select Protocol to Run
1_FirstStrandSynthesis.rst
- 2) Select PCR Plate labware for Thermal Cycling
96 ABI PCR half skirt in black carrier
- 3) Select Number of Columns of Samples
12
- 4) Select Input
Standard
- 5) Select Indexing Method
Single
- 6) Click button below to Display Initial Workstation Setup
- 7) Load labware according to Workstation Setup -->

Advanced Settings

- ☐ TESTING ONLY: Reduces all incubation times
- ☒ Play Alert Sound at User Prompts

Workstation Setup

MiniHub	MiniHub Cassette 1	MiniHub Cassette 2	MiniHub Cassette 3	MiniHub Cassette 4
Shelf 5				
Shelf 4				
Shelf 3				
Shelf 2	Empty Tip Box			
Shelf 1	Full Tip Box			Empty Tip Box

Bravo Deck

<Position 1>	<Position 2>	<Position 3>
<Pos 4: Peltier> 5 uL RNA in 96 ABI PCR half skirt in RED INSERT for FS1 rxn	<Pos 5: Shaker>	<Pos 6: Peltier> Empty 96 ABI PCR half skirt in RED INSERT for FS1 rxn
<Pos 7: Magnet>	<Position 8>	<Pos 9: Chiller> OFF MasterMix Eppendorf plate (Col 1, 2)

BenchCel

BenchCel Stacker 1	BenchCel Stacker 2	BenchCel Stacker 3	BenchCel Stacker 4
2 Tip Boxes	Empty	Empty	Empty

Controls

Once you have loaded labware according to Workstation Setup on right, click "Run Selected Protocol" to start run.

Information

Status

Figure 3. The VWorks interface for Lexogen QuantSeq enables easy and flexible automation of multiple desired workflows. The VWorks form has adjustable parameters that permit the user to run REV, FWD, and Flex kits from the same interface. On the left side, the user selects (1) which protocol step they would like to run, (2) what labware they would like to use, (3) the number of samples they would like to process, (4) the input amount, and (5) the indexing method. Once the parameters have been set, the user can click **Display Initial Workstation Setup** to update the central Workstation Setup display with the labware that needs to be placed on the instrument prior to running the protocol. Once the deck has been set up, the user can click **Run Selected Protocol** on the right side to start the protocol.

Materials and Methods

Before validating the NGS Workstation, the Bravo temperature settings, pipetting, and required dead volumes were optimized using the autoQuantSeq Dummy Automation Module (Lexogen), which provides liquids with the same physical properties as the standard reagent kits. After optimization using the Dummy Module, real reagents were used to verify pipetting accuracy.

To validate the performance of the NGS Workstation, UHRR (Agilent Technologies) libraries were prepared at two different input quantities using the QuantSeq 3' FWD kit. Eight 100 ng libraries and eight 250 ng libraries were prepared simultaneously with the NGS Workstation.

ERCC RNA Spike-in Mix (Thermo Fisher) was added to samples as per the manufacturer's protocol¹. Briefly, ERCCs were diluted 1:100 or 1:1,000, and 0.5 µL or 2 µL were added to each sample for 250 ng or 100 ng total RNA input, respectively.

The libraries were prepared using the NGS Workstation with VWorks software version 11.3.0.1195. The following parameters were used:

QuantSeq version	FWD
PCR Plate type	ABI MicroAmp Optical 96-well plate
Number of columns of sample	2
Input	Standard
Indexing	Single

The protocols used were as follows:

1. 1_FirstStrandSynthesis.rst
2. 2_SecondStrandSynthesis_Purification.rst
3. 3_LibraryAmplification.pro
4. 4_FinalPurification.pro

Reagent volumes, including the dead volume necessary for automation, were calculated from the reagent volume spreadsheet, which is linked from the VWorks form (Figure 4).

The 100 ng input samples underwent 20 cycles of amplification, and 250 ng input samples underwent 17 cycles of amplification. Final purified libraries were assessed on the Agilent Bioanalyzer using the High Sensitivity DNA assay, prepared according to manufacturer specifications². The libraries were pooled such that 20 fmol per sample (based on Bioanalyzer concentrations) was present in one pool and sequenced across two lanes of Rapid Run on an Illumina HiSeq 2500.

Sequencing reads were mapped using STAR³. ERCC correlations were calculated from \log_{10} ERCC-counts (number of reads) compared to \log_{10} ERCC-input (number of molecules) for each sample.

Lexogen QuantSeq 3' mRNA Library Prep on the Agilent Bravo

Please input values into yellow cells

SAMPLE PLATE: Provide 5 uL of RNA for each library, aliquotted in a PCR plate

Number of columns:	4	Input:	Standard	Tube Excess (uL):	10
Number of samples:	32	Indexing:	Single	Allows overage for creating master mixes in a 1.5 mL tube before transferring to automation master mix plate	

First Strand cDNA Synthesis

Reagent	Volume per Sample (uL)	Total volume (uL)	Excess for tube (uL)	Excess for plate (uL)	Excess %	Total volume to prepare (uL)	uL Buffer	uL Enzyme
FS1	5	160	N/A	24	15.00	184	184	0
FS2 / E1	10	320	10	24	10.63	354	336	18

Container	Volume per well (uL)
Eppendorf plate, column 1	23
Eppendorf plate, column 2	43

RNA Removal

Reagent	Volume per Sample (uL)	Total volume (uL)	Excess for tube (uL)	Excess for plate (uL)	Excess %	Total volume to prepare (uL)
RS1	5	160	N/A	24	15.00	184

Container	Volume per well (uL)
Eppendorf plate, column 3	23

Second Strand Synthesis

Reagent	Volume per Sample (uL)	Total volume (uL)	Excess for tube (uL)	Excess for plate (uL)	Excess %	Total volume to prepare (uL)	uL Buffer	uL Enzyme
SS1	10	320	N/A	28	8.75	348	348	0

Container	Volume per well (uL)
Eppendorf plate, column 4	44
Eppendorf plate, column 5	44

QuantSeq FWD and REV QuantSeq Flex MasterMix Plate Layout Dummy Reagents + 4

Figure 4. Reagent volumes have been precalculated with the reagent spreadsheet. The form (see Figure 3) links to a reagent volume spreadsheet. The user enters parameters such as the number of samples and input amount, and the spreadsheet outputs how much of each reagent to prepare, including dead volume for both manual and Agilent Bravo pipetting steps.

Results and Discussion

The Bioanalyzer results show fragments between 135 and 500 base pairs (bp) with a peak at approximately 205 to 225 bp (Figure 5). The Bioanalyzer-estimated concentrations

range from 5.8 to 11.3 ng/ μ L for the 100 ng input samples and from 3.1 to 12.1 ng/ μ L for the 250 ng input samples (Table 1). The samples were of consistent fragment sizes, high concentration, and comparable to manually-produced libraries.

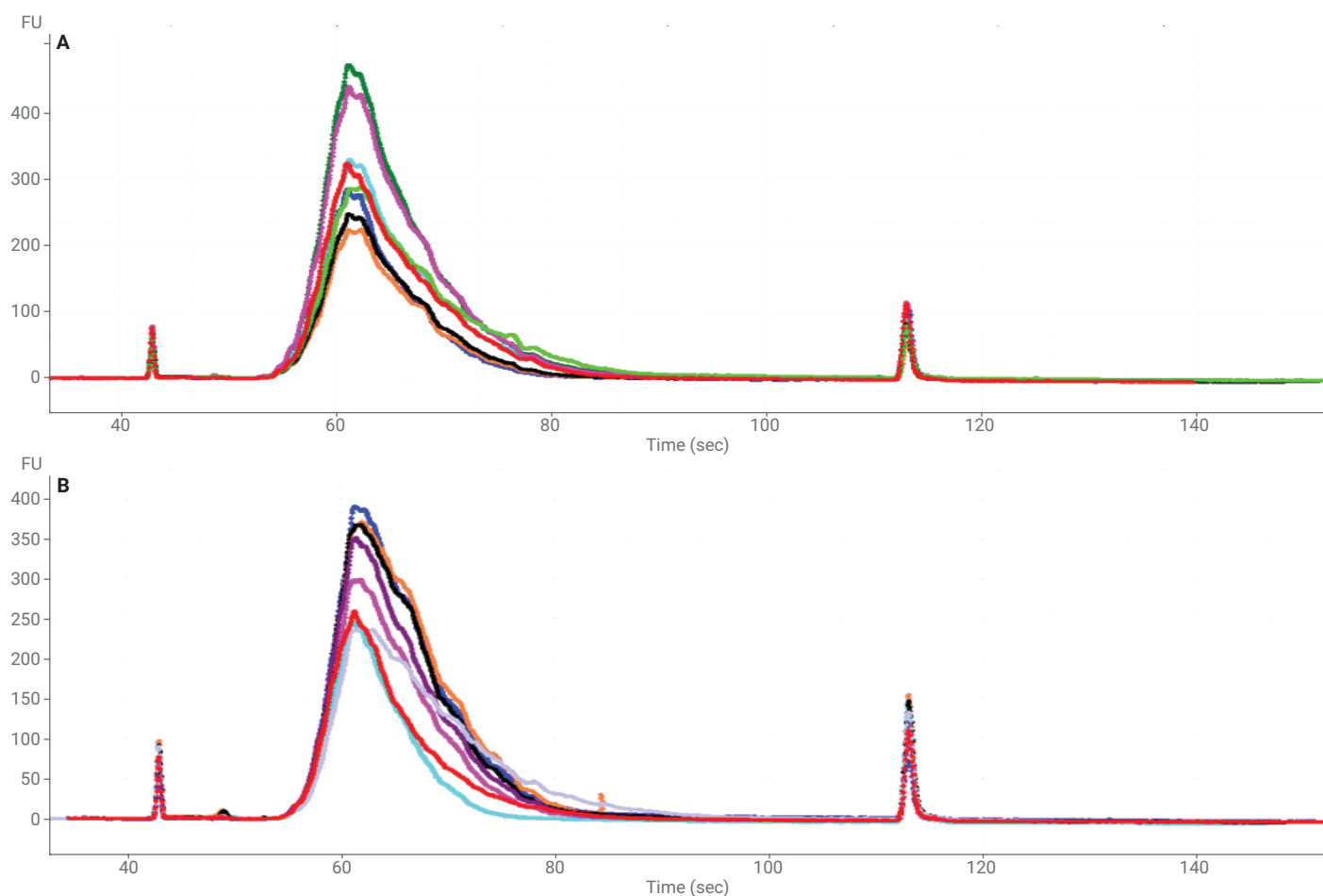


Figure 5. Bioanalyzer results show consistency across automated samples. The final libraries were analyzed using the Agilent High Sensitivity DNA Bioanalyzer assay. The libraries produced from the 100 ng input are displayed in (A), and the libraries produced with 250 ng input are displayed in (B). The electropherograms show reproducible size distribution and concentrations. Note that some samples within a group were run on separate Bioanalyzer chips; therefore, it is impossible to display the x-axis in base pairs. The 60-second tick mark corresponds to approximately 200 bp.

Table 1. Universal human reference RNA (UHRR) was used as input to the QuantSeq method. Sixteen samples were processed on the Agilent NGS Workstation, eight using 250 ng and eight using 100 ng input. The final library yields range from 3.1 to 12.1 ng/μL.

Sample ID	Input (ng)	Number of PCR cycles	Yield (ng/μL)
A1	100	20	7.2
A2			5.8
A3			11.3
A4			8.9
A5			10.1
A6			9.0
A7			7.0
A8			9.9
A9	250	17	5.1
A10			12.1
A11			3.1
A12			6.2
A13			6.3
A14			6.0
A15			7.1
A16			5.4

Table 2 provides the results from the sequencing data analysis. On average, the samples had 9.5 million reads each. The 100 ng samples had an average of 4.9 million reads each, while the 250 ng samples had an average of 14 million reads each. This variance in read counts may be due to the use of the Bioanalyzer for quantification before pooling. The 250 ng samples had a higher percentage of uniquely mapped reads, which is to be expected from the higher input. Additionally, the 250 ng samples had a lower proportion of rRNA (2.69 % ± 0.8) compared to the 100 ng samples (7.33 % ± 2.4).

Table 2. Libraries prepared with the Agilent NGS Workstation produce good quality sequencing data. The number of reads per library ranged from 3,130,794 to 29,917,053. On average, the libraries prepared with 250 ng input had higher uniquely mapped reads, a lower percentage of rRNA, and a higher number of detectable ERCC transcripts. Both inputs produced high-quality data.

Sample ID	Number of reads	Uniquely mapped reads (%)	rRNA (%)	Number of ERCC transcripts detected
A1	3,442,286	84.4	3.13	61
A2	6,546,644	78.7	9.40	60
A3	3,130,794	82.3	5.21	58
A4	5,708,823	81.2	6.66	63
A5	3,754,829	80.7	7.31	58
A6	4,006,940	77.9	10.87	63
A7	8,225,667	80.1	7.65	67
A8	4,683,424	78.8	8.44	62
A9	7,906,501	85.4	1.89	69
A10	6,042,257	84.0	3.20	70
A11	10,750,477	83.6	2.76	76
A12	12,295,976	85.1	2.04	70
A13	13,155,973	83.8	3.47	73
A14	29,917,053	83.4	3.90	77
A15	9,352,055	84.6	2.57	70
A16	22,481,940	85.7	1.65	75
Overall average	9,462,602	82.5	5.01	67
100 ng Average	4,937,426	80.5	7.33	62
250 ng Average	13,987,779	84.4	2.69	73

On a per sample basis, the number of reads mapping to each ERCC transcript was correlated with the number of molecules of that transcript in the sample. Of the 92 unique ERCC transcripts in each sample, an average of 62 were detected in the 100 ng samples, and 73 in the 250 ng samples. For both, transcripts that were not detected were present at the lowest concentrations (data not shown). The Pearson correlations are plotted in Figure 6. The data show that ERCC input and output are highly correlated across all samples. At the 250 ng input level, the average correlation was slightly higher (0.964 ± 0.011) than the 100 ng input level (0.957 ± 0.008).

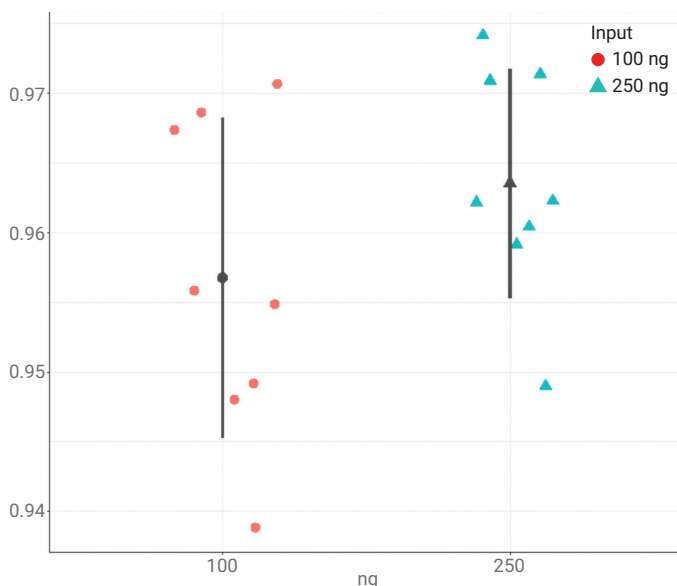


Figure 6. ERCC input and output are highly correlated across samples. The number of reads mapped to ERCCs was correlated with their concentration in each sample. The average Pearson correlation for 100 ng input libraries was 0.957. The average Pearson correlation for 250 ng input libraries was 0.964. In the plot, the red circles represent the 100 ng samples, and the blue triangles represent the 250 ng samples. The gray circle and gray triangle correspond to the means, and the gray bars represent the standard deviation.

Conclusion

The implementation of the Lexogen QuantSeq 3' mRNA protocol on the Agilent NGS Workstation has been shown to produce high-quality sequencing libraries. The resultant libraries, from two different input amounts, are of consistent size, good concentration, high unique mappability, low rRNA percentage, and high correlation between ERCC input and output. The NGS Workstation allows production of up to 96 libraries in 8.5 hours, with only 2 hours of hands-on time (total and hands-on time is lower with lower sample volumes). The automation protocol is easy to implement, and allows flexible automation of the many different types of QuantSeq kits.

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For More Information

Contact Agilent for a copy of the QuantSeq NGS Workstation protocol.

www.agilent.com/chem

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