BECKMAN COULTER
Life Sciences

Automation of New England BioLabs NEBNext Ultra II Directional RNA Library Preparation Kit for Illumina on Beckman Coulter Biomek NGeniuS Next Generation Library Prep System

Introduction

As genome sequencing and data analysis methods become more accessible, more laboratories are exploring NGS (Next Generation Sequencing) as a research tool. Laboratories are looking for reproducible NGS sample prep methods that limit potential for in-process sample degradation or error. In this paper, we detail an automated process for the New England Biolabs NEBNext Ultra II Directional RNA Library Preparation Kit for Illumina that offers the laboratory optional settings to optimize a demonstrated application that will process between 4 and 24 samples from start to finish, with minimal interaction from the user. Library preparation results using the NEBNext Ultra II Directional RNA Library Preparation Kit on the Biomek NGeniuS Library Prep System indicate an average of 98% of reads align with the reference transcriptome.

Introduction

In the past 20 years, sequencing methods have changed drastically. The completion of the Human Genome Project in 2003 heralded a new era in biomedical research: not only did researchers have more knowledge about the human genome than ever before; but also had mature computer programs to analyze data and assist in discovering the interactions between changes in genetic information and disease states. Despite these advances, the cost to sequence was still prohibitive to obtain degrees of coverage required for clinicians to be certain of linkages between genotype and disease phenotype. In 2005, massively parallel sequencing (or Next-Generation Sequencing, referred to as “NGS”) was introduced to the scientific community. The process involved making a library of DNA fragments that were able to be traced back to the original sample by a “barcode.” These systems allowed up to 700 bp of sequence to be sequenced overnight in millions of different samples. Data analysis programs developed alongside massively parallel sequencing allowed for analysis of the large volumes of information being created.

A routine part of sequencing workflows is the generation of sequencing libraries, but the creation of libraries for NGS can be a tedious process, taking anywhere from 2.5 hours to several days to complete depending on the kit. Great care must be taken by the user during library preparation and data handling to ensure that the correct adaptor sequences and indices are added to the corresponding samples. Also, many preparation workflow steps require careful timing and do not have safe stopping points, leading to a very long day for the user. Because of these concerns, the automation of NGS library preparation kits using liquid handling systems like the Biomek NGeniuS is highly desirable (Figure 1). One such kit is the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina.

The NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina® for Poly(A) mRNA Magnetic Isolation Module provides high-quality reproducible libraries from total RNA for direct sequencing, or as an input for hybridization capture assays. General workflow steps for this kit are outlined in Figure 1. Briefly, sample inputs must contain between 1-100 ng of purified mRNA or rRNA depleted RNA quantified after purification. This kit is optimized for RNA fragments 200 bp in length. RNA samples must be free of DNA, salts or chelating agents. mRNA samples are bound to Oligo dT beads, then washed and eluted in Tris buffer. RNA samples are then thermally fragmented and immediately reverse transcribed to double-stranded cDNA. Double-stranded cDNA is cleaned up and size selected for the target fragment size. Fragment ends are prepared and adaptors ligated. Finished adaptor ligation reactions are purified, washed and eluted. Fragments are then PCR amplified using supplied forward and reverse (i7/i5, respectively) primers. PCR cycling parameters vary based on sample starting concentrations. Finally, PCR reactions are cleaned up and assessed for quality. Libraries are expected to be approximately 300 bp[1]. In this application note, we demonstrate that automated processing on Biomek NGeniuS is equivalent to the manual preparations for RNA input concentrations of 10, 100 and 1000 ng. This reduces hands-on time for processing, user interactions/touch points, and handling errors.

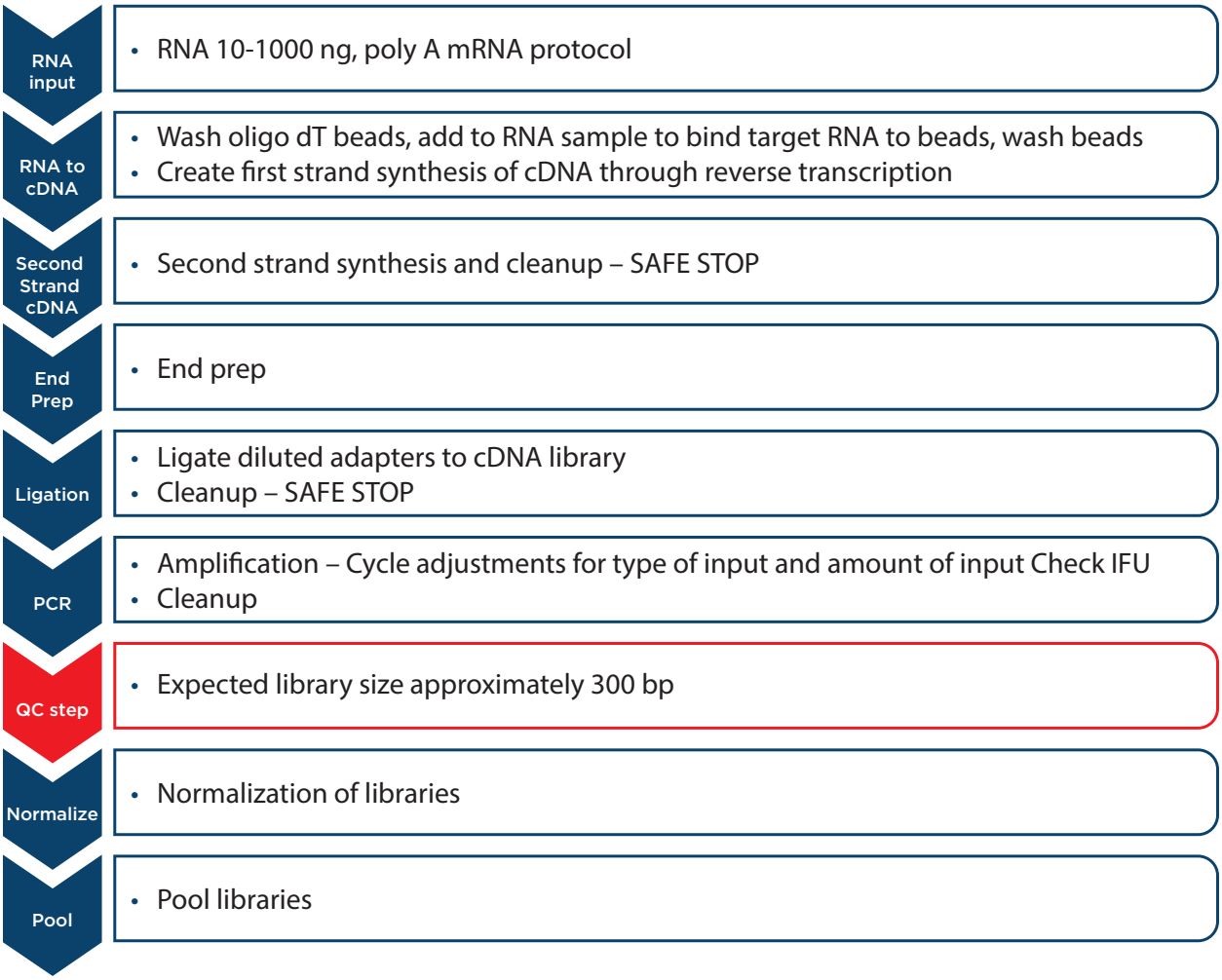


Figure 1. Workflow for NEBNext Ultra II Directional RNA, poly A mRNA isolation from total RNA protocol for demonstration purposes. (Blue- On system, Red- Off system)

Methods

1. Run Setup

RNA samples (Table 1) were diluted to 10, 100, or 1000 ng inputs according to library prep instructions. Once samples were ready for library preparation, the Biomek NGeniuS system's customer portal was used to prepare the instrument. The first step was to select the +create button to create a batch to be run on the system (Figure 2). Next, the App for the NEBNext Ultra II Directional RNA Kit was selected to process samples. The setup is broken into four sections: Batch Info (name of batch and number of samples to be run), App Settings, Sections and Sample Data (Figure 3). App Settings contains variables specific to the library kit that may be changed between runs or locked by an administrator. The Batch name is a unique run name for the samples being processed. Number of samples is between 4 and 24 for this application, as indicated by the light grey numbers below the input box. Table 2 lists the app settings and descriptions of each setting.

Figure 2. The **+create** button is used to begin a new batch setup.

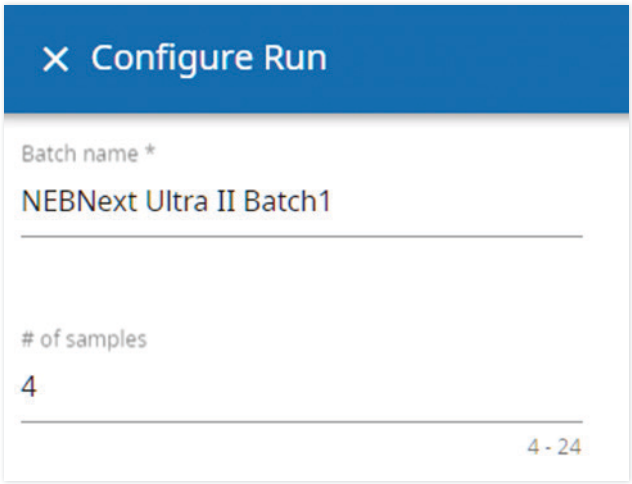
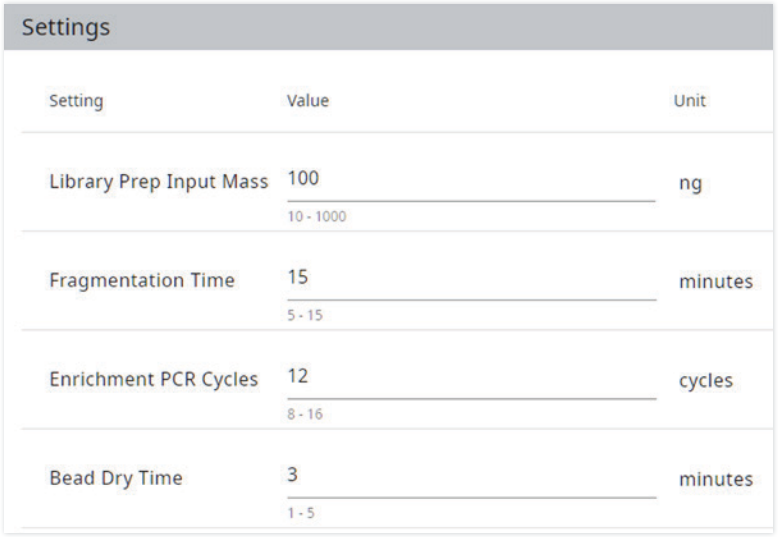


Figure 3. Batch information and default applications settings for batch run.



Sample	Vendor	Part Number
Universal Human Reference RNA	Agilent Technologies	740000

Table 1. Sample types and inputs used in preparations of samples for NEB Next Ultra II RNA library Prep.

Setting	Description
Library Prep Input Mass	The starting mass of RNA in a sample for library preparation. In this application the weight is recommended to be between 1-100 ng of RNA.
Fragmentation Time	The time elapsed while RNA samples are fragmented at 94ºC. Incubation time varies by RIN value.
Enrichment PCR Cycles	The number of PCR enrichment cycles. In this application the number is between 5-12 cycles, depending on sample input mass.
Bead Dry Time	The amount of time beads with attached libraries dry after a post-PCR cleanup ethanol wash. In this application the dry time is between 1-5 minutes, never exceeding 5 minutes.

Table 2. App Settings for the NEBNext Ultra II Directional RNA library Preparation Kit and descriptions for each setting.

The next section of data to be filled out is **Sections** (Figure 4). The NEBNext Ultra II Directional RNA Library Prep Kit has four potential sections. Users can select where to start in the process just below the Sections marker in Figure 4. Some users may prefer to do the first section, **Normalize Samples**, by hand. If so, they can elect to utilize the **Start at Section** dropdown menu to select **cDNA Synthesis** as the library prep starting point. A drop-down menu allows the user to select any of the four Sections as the starting point. The starting points are determined by safe stops defined in the instructions for use in each library prep kit, which are also suitable for a safe stop on the Biomek NGeniuS system. The blue slider to the left of the Sections allows the user to select a safe stop to end processing of the samples. The instrument is designed to be run unattended, but users can elect to stop processing and store samples safely before resuming in the run at the next shift.

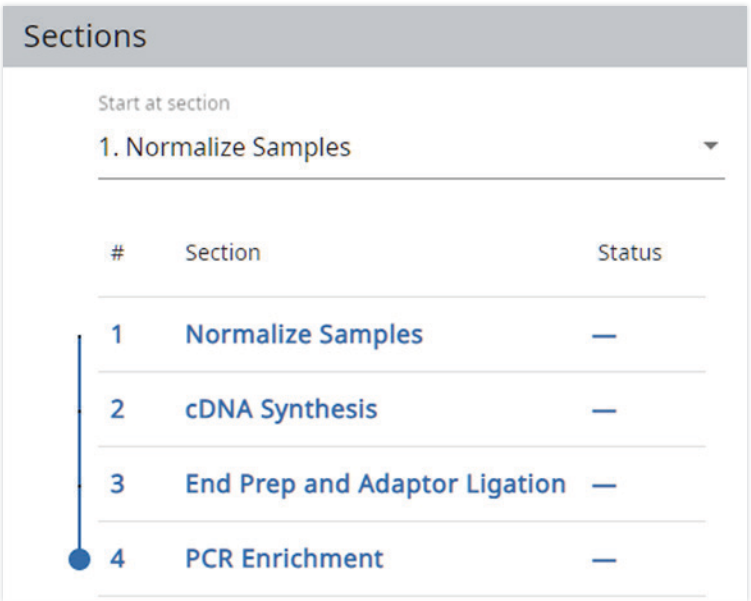


Figure 4. Sections for NEBNext Ultra II RNA Application.

The final step in setting up a batch run is to input the sample data (Figure 5). In the sample data section, users can click the **DOWNLOAD SAMPLE DATA TEMPLATE** and fill in the appropriate information. This is a .csv file that is filled out and uploaded into the sample data by clicking the **Upload** button. Users can utilize tool tips to determine what information goes into each column by hovering over the header of each column in the NGeniuS Portal. The NEBNext Ultra II RNA Application requires the user to define four data pieces for each sample. The first user defined column is the **Sample_ID** of each sample. The second item, **Index i5**, is the reverse index plate's well to be associated with that sample, while the second item, **Index i7** corresponds to the forward index plate. The final column, **Initial Concentration**, is the concentration of RNA that is to be placed into each well for dilution and processing for library preparation. Once data is entered in the template and saved, the user clicks the **Upload** button. If there are any errors in the sample data file, a red box will appear indicating the source of the problem. Users can fix the data file and upload again if needed. The final step is to click the **READY TO RUN** button in the top right of the screen. The batch can be initiated at any Biomek NGeniuS system within the same tenant.

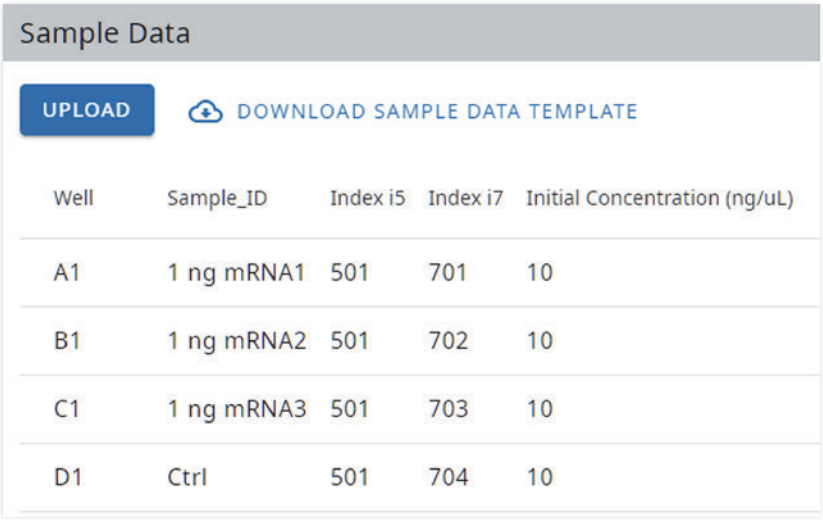


Figure 5. Simulated **Sample Data** information. The four columns starting with **Sample_ID** must be user defined in the sample-data-template.csv file and uploaded to the Biomek NGeniuS Portal.

Reagents	Manufacturer	Part Number
Poly A mRNA magnetic isolation module, 96 reactions	New England Biolabs	E7490L
NEB Next Ultra II Directional RNA library prep kit for Illumina, 96 reactions	New England Biolabs	E7765L
Qubit High Sensitivity Kit	Thermo	Q32854
Bioanalyzer High Sensitivity Kit	Agilent	5067-4627
NextSeq Sequencing Kit	Illumina	20024907
AMPure XP Beads	Beckman Coulter	A63882
PCR grade Water	Invitrogen-Life Technologies	10977-023
Ethanol, 100%	American Bio	AB00515-0500

Table 3. Reagents used in preparation of libraries with NEB Next Ultra II RNA library Prep and sequencing on Illumina sequencer.

Equipment	Manufacturer
Biomek NGeniuS Next Generation Library Prep System	Beckman Coulter
NextSeq 550 Sequencer	Illumina
Allegra X-14 Centrifuge	Beckman Coulter
Qubit Fluorometric Quantification system	Thermo Fisher Scientific
Agilent 4200 TapeStation	Agilent

Table 4. Equipment used in sample preparation and processing NEB Next Ultra II RNA library Prep.

Consumable	Manufacturer
Qubit Tubes - Q32856	ThermoFisher Scientific
NGeniuS 24 well plates/lids – C62706	Beckman Coulter
NGeniuS Bulk reservoirs – C62707	Beckman Coulter
NGeniuS seal pads – C70665	Beckman Coulter
NGeniuS reagent plugs	Beckman Coulter

Table 5. Consumables required for sample processing.

Sample Type	Library Prep Input Mass (ng)	Fragmentation Time (min)	Enrichment PCR Cycles (min)	Bead Dry TIme (min)
RNA	10 ng	15	15	3
RNA	100 ng	15	12	3
RNA	1000 ng	15	8	3

Table 6. Method variables and selections for NEBNext Ultra II Directional RNA Library Prep kit. Parameters outlined in this table correspond to selections made in "Settings" when initiating a run (Figure 3).

2) Library Preparation

Samples of *Homo sapiens* Universal Reference RNA (Table 1) were processed on the Biomek NGeniuS system using reagents, equipment and consumables detailed in Tables 3, 4, and 5. Input samples were diluted to starting concentrations appropriate for a max of 1:100 dilution of ample prior to preparing the library. This was determined by taking the library prep input mass (in ng) and dividing by the input volume in µL. System requested reagents (contained within the NEB Ultra II Directional RNA kit and bulk reagents [Table 3]) and Biomek NGeniuS consumables (Table 5) were loaded onto the system for processing. The variables selected for both manual and automated processing were as seen in Table 6. After all reagents and consumables had been allocated to proper indicated storage locations, the user was instructed to remove reagents and notified of an estimated time of completion for the library prep based off selections the user input at the start of the protocol. The system processed dilution of samples. Samples were processed and libraries were constructed on the system. After completion of the runs, the constructed libraries were analyzed by traces on an Agilent 4200 TapeStation system (Figure 6), DNA fluorometric yields (Table 7), and Illumina sequencing (Table 8).

Library Starting Mass	Qubit Yield (ng/µL)
10 ng	8.37
100 ng	8.75
1000 ng	14.93

Table 7. Average Qubit Yields for library preparations for across libraries prepared with the NEBNext Ultra II Directional RNA Library Preparation Kit.

Results and Discussion

After completion of the runs by the Biomek NGeniuS NGS Library Prep System, libraries were sequenced using an Illumina NextSeq 1000 instrument using High Output v3 reagent kits (Illumina, Inc.). Fragment size was measured with an Agilent 4200 TapeStation system (Agilent Technologies, Inc.)., and library prep yield masses were measured with Qubit fluorometric quantification (Thermo Fisher). Sequencing results returned 484M reads. Sequencing results were scored greater than Q30 for 93% of bases.

24 libraries (one negative control) were prepared from 10 ng *Homo sapiens* reference RNA samples (Agilent Technologies, Inc.). Libraries returned average masses of 8.37 ng/µL. Results indicated a consistent and normal distribution of read lengths, with the average library size being 420 bp (Figure 6a). 97.85% of reads were aligned to reference transcripts. An average of 7.4% of reads across 23 samples were duplicates (Table 8).

Seven libraries (one negative control) were prepared from 100 ng *Homo sapiens* reference RNA samples (Agilent Technologies, Inc.). Libraries returned average masses of 8.75 ng/µL. Results indicated a consistent and normal distribution of read lengths, with the average library size being 432 bp (Figure 6b). Of this sample, 98.41% of reads were aligned to the reference transcripts. An average of 3.68% of reads across five samples were duplicates (Table 8).

Four libraries (one negative control) were prepared from 1000 ng *Homo sapiens* reference RNA samples (Agilent Technologies, Inc.). Libraries returned average masses of 14.93 ng/µL. Results indicated a consistent and normal distribution of read lengths, with the average library size being 441 bp (Figure 6c). Of this sample, 98.42% of reads were aligned to reference transcripts. An average of 4.73% of reads across four samples were duplicates (Table 8).

Input DNA Mass	Sample Type	Average Library Size (bp)	Qubit Conc. (ng/µL)	% >Q30	% Duplicates	Average % Reads Aligned	Average % Stranded
10 ng	RNA	420	8.37	93	7.4%	97.85%	99.4%
100 ng	RNA	432	8.75	93	3.68%	98.41%	99.42%
1000 ng	RNA	441	14.93	93	4.73%	98.42%	99.61%

Table 8. Sequencing results from the NEBNext Ultra II Directional RNA Library Prep Kit on the Biomek NGeniuS Library Prep System. Results fall within library parameters defined by NEB.[1]

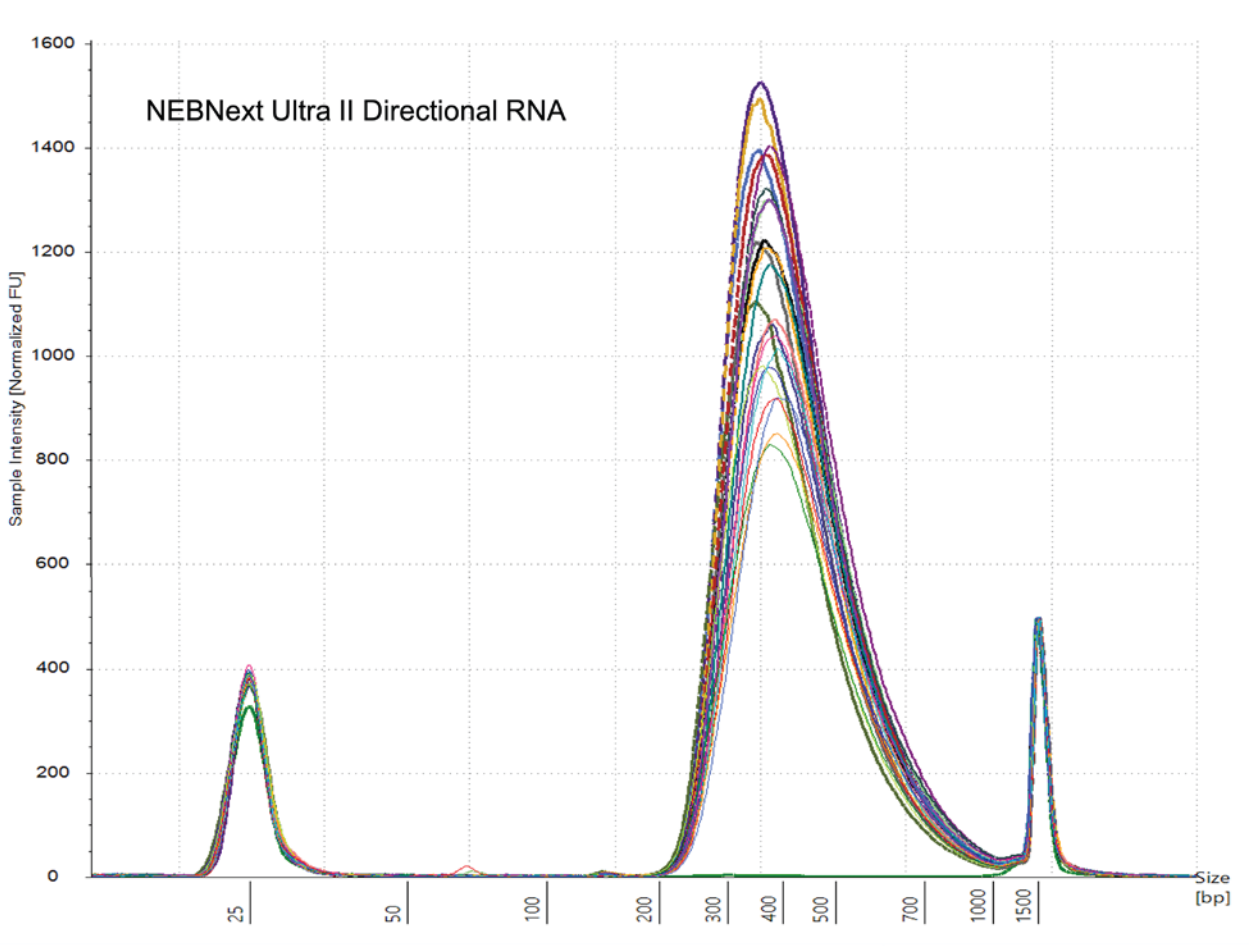


Figure 6a. Agilent TapeStation trace results from the libraries created on the Biomek NGeniuS Next Generation Library Prep System from 23 reference RNA samples and one negative control using the NEBNext Ultra II Directional RNA Library Prep Kit. Libraries have an average fragment size of 420 bp, averaged across all libraries created from these samples.

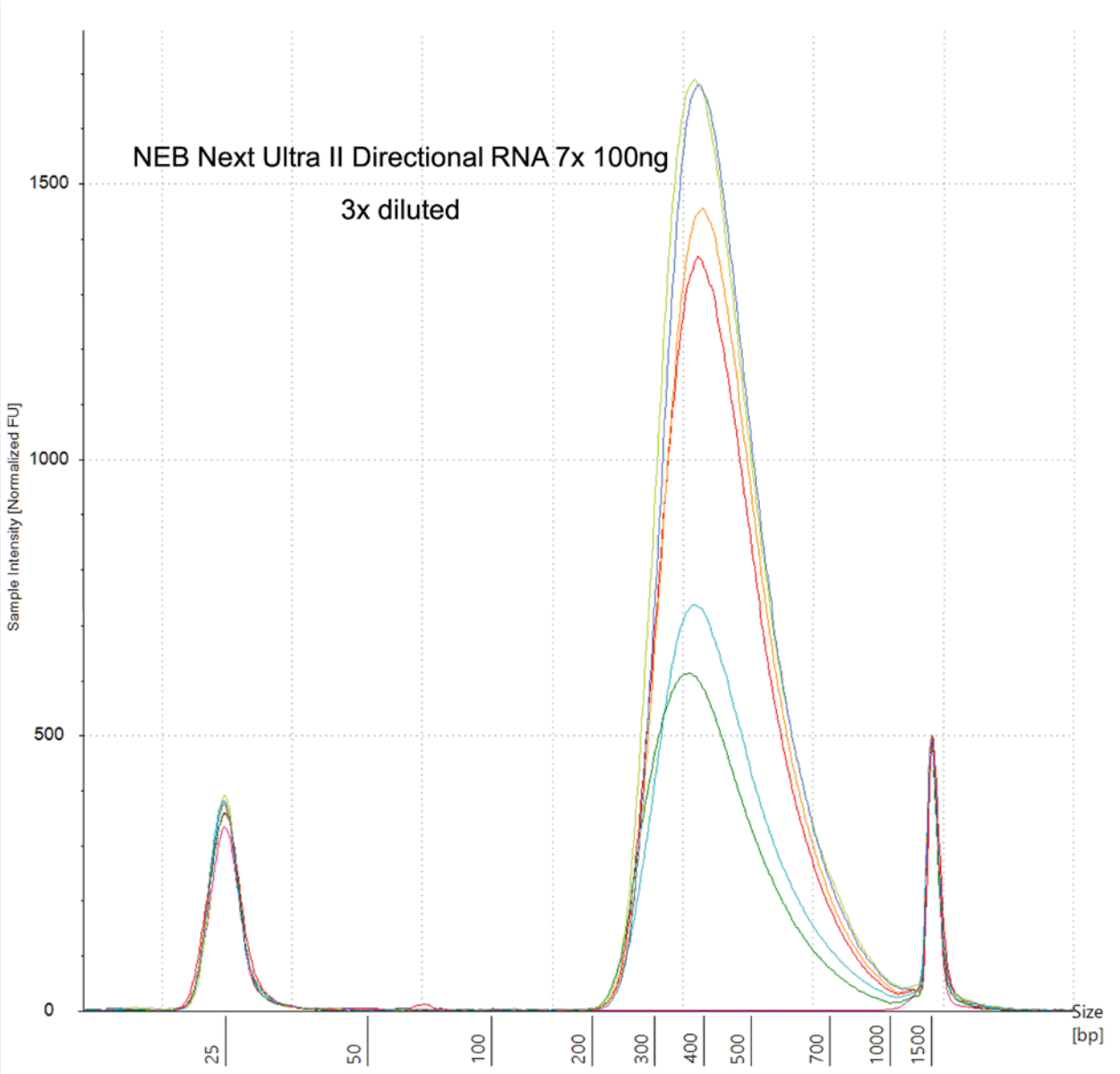


Figure 6b. Agilent TapeStation trace results from libraries created on the Biomek NGeniuS next generation library prep system from 6 reference RNA samples and one negative control using the NEBNext Ultra II Directional RNA Library Prep Kit. Libraries have an average size of 432 bp per fragment, averaged across all libraries created from these samples.

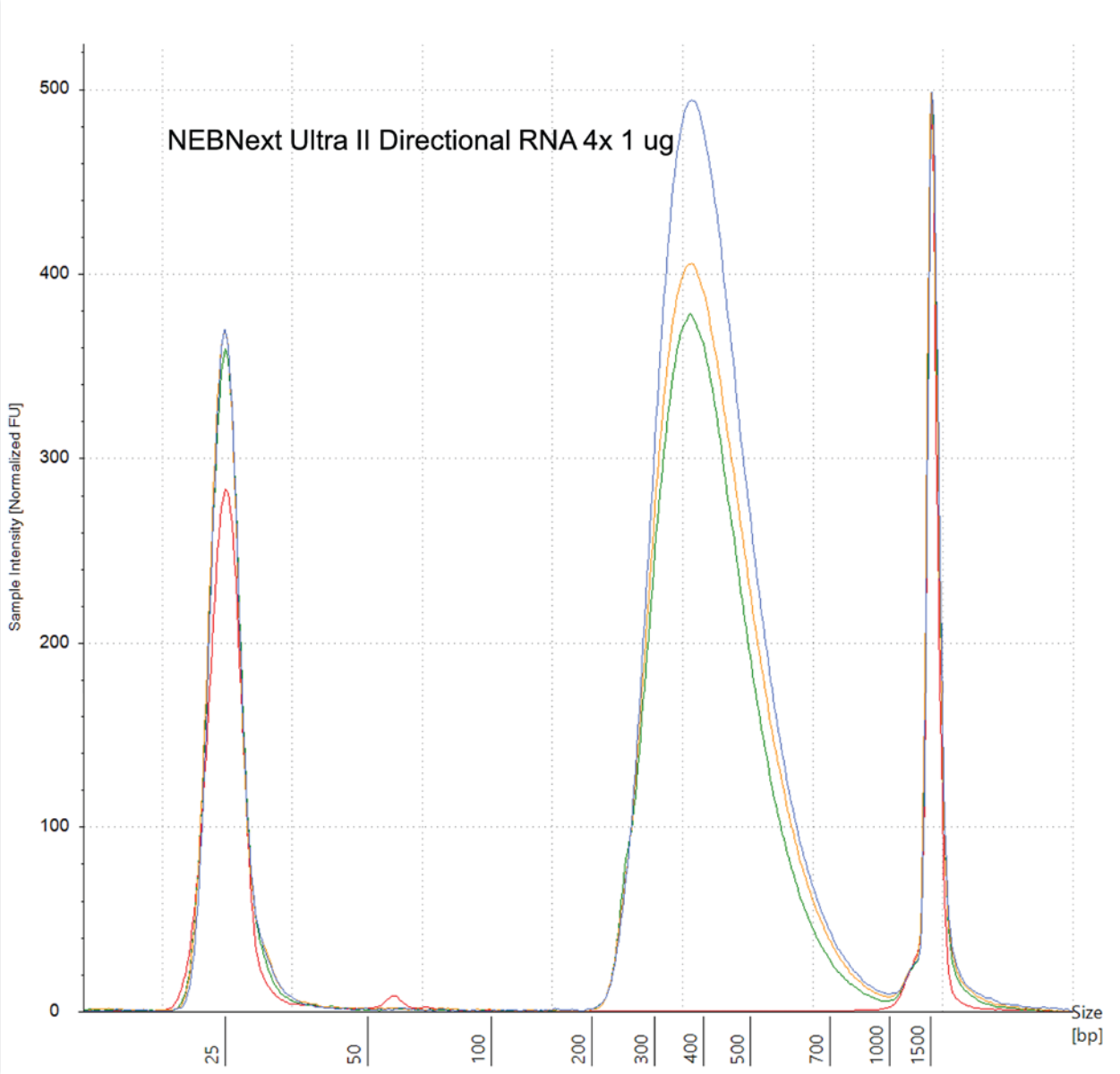


Figure 6c. Agilent TapeStation trace results from libraries created on the Biomek NGeniuS next generation library prep system from 3 reference RNA samples and one negative control using the NEBNext Ultra II Directional RNA Library Prep Kit. Libraries have an average size of 441 bp per fragment, averaged across all libraries created from these samples.

Summary

Libraries generated using the NEBNext Ultra II Directional RNA Library Prep Kit on the Biomek NGeniuS Next Generation Library Prep System show uniform size distributions on the Agilent 4200 TapeStation system (Figure 6) and fall within the recommended library size range of the NEBNext Ultra II Directional RNA Library Prep Kit. Sequencing of the replicates of samples for three difference concentrations produced average library sizes in a range between 420-441 bp. Across all samples processed, over 97.85% of reads were aligned to reference transcripts and less than 7.4% of reads were duplicates (Table 8).

We demonstrated that the Biomek NGeniuS Next Generation Library Prep System can successfully produce high-quality RNA-Seq libraries suitable for sequencing on the Illumina platform from RNA samples using the NEBNext Ultra II Directional RNA Library Prep Kit.

References

1. Protocol for use with NEBNext Poly(A) mRNA Magnetic Isolation Module and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina.

For Beckman Coulter's worldwide office locations and phone numbers, please visit Contact Us at beckman.com

22.05.5041.GEN