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Automation of New England BioLabs NEBNext Ultra II Directional RNA Library Preparation Kit for Illumina with Plate-based Unique Dual Indices on the Biomek NGeniuS Next Generation Library Prep System

Abstract

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 Prep Kit for Illumina® using the NEBNext® Poly(A) mRNA Magnetic Isolation Module and plate-based Unique Dual Index primers. The Biomek NGeniuS Library Prep System 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. Sequencing results from library preparation indicate more than 97% 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 cumbersome 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 adapter 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 handlers like the Biomek NGeniuS system is highly desirable **(Figure 1)**. One such library preparation kit is the NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina®.

The NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina® with NEBNext® 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 10-1000 ng of total RNA quantified after purification. This kit is optimized for RNA inserts 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 adapters ligated. Finished adapter 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 the **NEBNext Ultra II Directional RNA polyA for IndexPlate** App on the Biomek NGeniuS system for RNA input concentrations of 10, 100 and 1000 ng, and we have also sequenced the libraries and analyzed data obtained from processing using the instrument.

Materials and Methods

1.1 Run Setup

RNA samples **(Table 1)** were diluted to 10, 100, or 1000 ng inputs according to library prep instructions.

Sample	Vendor	Part Number
Universal Human Reference RNA	Agilent Technologies	740000

Table 1. Sample types used in preparations of samples for NEBNext Ultra II Directional RNA library Prep.

Normalization of input nucleic acid is performed on the instrument by diluting an aliquot of the sample to the input volume required by the library preparation kit to arrive at the correct starting concentration.

To reduce manual pipetting errors, the concentration of input nucleic acid must be within 100X of the concentration required by the library preparation kit so that the operator is not attempting to manually pipette small volumes of highly concentrated input nucleic acid.

When samples are ready, the run is set up in the Biomek NGeniuS customer portal. The **NEBNext Ultra II Directional RNA polyA for IndexPlate** App has settings which allow the operator to select the library prep input mass, fragmentation time, first strand synthesis incubation time, purification bead dry time, index plate set, and enrichment PCR cycles. A summary of experiment details and parameters is presented in Table 2. All experiments used the 96 Unique Dual Index Primer Pairs Set 1, 96-well index plate.

Experiment	1	2	3
Sample count	4	9	24
Library Prep Input Mass (ng)	1000	100	10
Fragmentation Time (min)	15	15	15
1st Strand Incubation Time (min)	15	15	15
Bead Dry Time (min)	3	3	3
Enrichment PCR Cycles	8	12	15

Table 2. Summary of experiment conditions and App Settings. All experiments used the 96 Unique Dual Index Primer Pairs Set 1, 96-well index plate and included 1 negative control.

The App allows for individual sections (corresponding with safe stopping points) to be run in isolation. For the 4- and 9-sample experiments, the App was run straight through. For the 24-sample experiment, Normalization and cDNA Synthesis were run separately from End Prep and Adapter Ligation and PCR Enrichment sections due to needing to refresh consumables.

Figure 1. Workflow for NEBNext Ultra II Directional RNA Library Prep protocol on the Biomek NGeniuS system. The blue arrows indicate the steps that are done on the instrument; the red arrows indicate the steps that are not.

1.2 Reagents, Consumables, and Equipment

Sample	Vendor	Part Number
NEBNext Ultra II Directional RNA Library Prep kit for Illumina, 96 reactions	New England Biolabs	E7765L
Poly A mRNA magnetic isolation module, 96 reactions	New England Biolabs	E7490L
NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs), Set 1	New England Biolabs	E6440S
Qubit 1X dsDNA HS Assay Kit	Thermo Fisher Scientific	Q33231
High Sensitivity D1000 ScreenTape	Agilent	5067-5584
High Sensitivity D1000 Ladder, Reagents	Agilent	5067-5587, 5067-5585
NextSeq 1000/2000 P2 Reagents (300 Cycles) v3	Illumina	20046813
PCR grade Water	Invitrogen-Life Technology	10977-015
Ethanol	Thermo Fisher Scientific	BP2818-500

Table 3. Reagents used in preparation of libraries with NEBNext Ultra II Directional RNA Library Prep and sequencing on Illumina sequencer.

Equipment	Manufacturer
Biomek NGeniuS Sample Prep System	Beckman Coulter Life Sciences
NextSeq 1000 Sequencer	Illumina
Qubit Fluorometer	Thermo Fisher Scientific
4200 TapeStation System	Agilent

Table 4. Equipment used in sample preparation and processing of NEBNext Ultra II Directional RNA Library Prep.

Consumable	Manufacturer/ Part Number
Foil Plate Seals	Beckman Coulter 538619
Biomek NGeniuS Reaction Vessel, 24 Well	Beckman Coulter C62705
Biomek NGeniuS Lid, 24 Well	Beckman Coulter C62706
Biomek NGeniuS Bulk Reservoirs, 25 mL/Section	Beckman Coulter C62707
Biomek NGeniuS Seal Pad	Beckman Coulter C70665
1025 µL Conductive Filtered Tips, Case	Beckman Coulter C59585
70 µL Conductive Filtered Tips, Case	Beckman Coulter C62712
Empty Tip box 1025 µL, Case	Beckman Coulter C70672
Empty Tip box 70 µL, Case	Beckman Coulter C70673

Table 5. NGeniuS consumables required for sample processing.

1.3 NGeniuS System Produced Libraries and Sequencing

Samples of *H. 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. After completion of the runs, the resulting libraries were analyzed using the 4200 TapeStation with D1000 High Sensitivity ScreenTape (Agilent) to determine library size, and the Qubit 1X dsDNA HS assay kits (Thermo Fisher Scientific) to determine library concentration.

Libraries were then pooled and sequenced on an Illumina NextSeq 1000 system using a NextSeq 1000/2000 P2 Reagents (300 Cycles) v3 with a 2x100 bp sequencing run and a loading concentration of 750 pM. The 100 and 1000 ng runs were pooled and run together, while the 10 ng run was pooled and run independently.

Data were analyzed using BaseSpace DRAGEN Analysis version 1.3.0, RNA-Seq Alignment version 2.0.2, and DRAGEN RNA version 4.0.3 Apps on Illumina BaseSpace. For RNA-Seq, the Homo sapiens (PAR-masked)/hg19 reference genome provided by BaseSpace was used. For DRAGEN RNA, the Human (UCSC HG19 Alt-Masked V2) reference genome provided by BaseSpace was used, and libraries were down-sampled to 4 million reads (2 million each direction).

Results & Discussion

The 10 ng input RNA run produced 23 libraries (plus one negative control) with an average size of 321 bp **(Figure 2)**. The average yield, as measured by Qubit, was 7.40 ng/mL. The 100 ng input RNA run produced 8 libraries (plus one negative control) with an average size of 337 bp **(Figure 3)**. The average yield, as measured by Qubit, was 10.41 ng/mL. The 1000 ng input RNA run produced 3 libraries (plus one negative control) with an average size of 327 bp **(Figure 4)**. The average yield, as measured by Qubit, was 7.35 ng/mL. All negative controls were devoid of library at the expected ~300 bp fragment size.

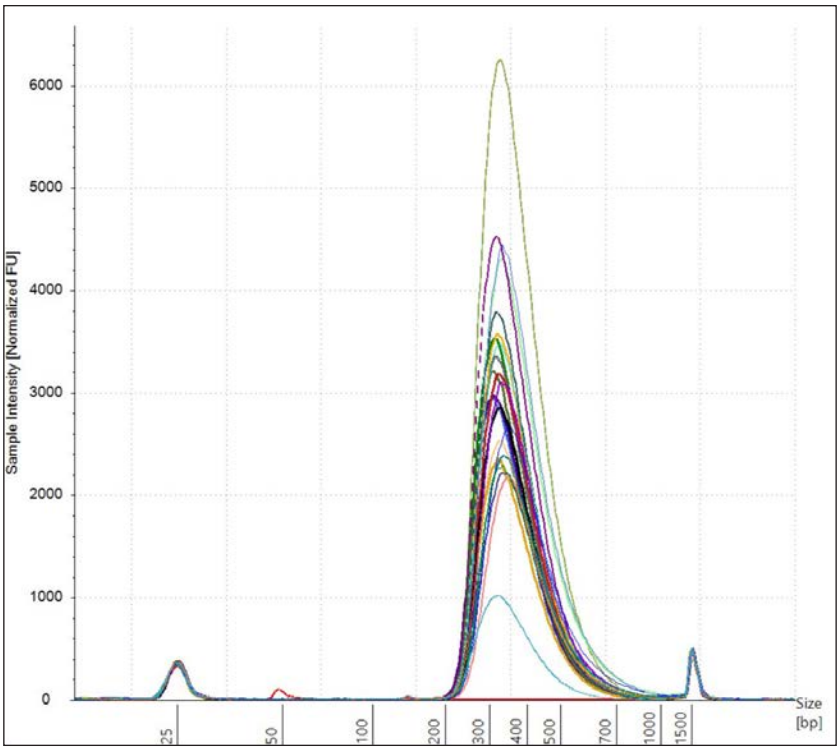

Figure 2. Agilent TapeStation trace results from libraries created on the Biomek NGeniuS system from 23 RNA samples (10 ng input mass) and one negative control using the NEBNext Ultra II Directional RNA Library Prep kit. Libraries have an average fragment size of 321 bp, averaged across all libraries created from these samples. Red trace is negative control.

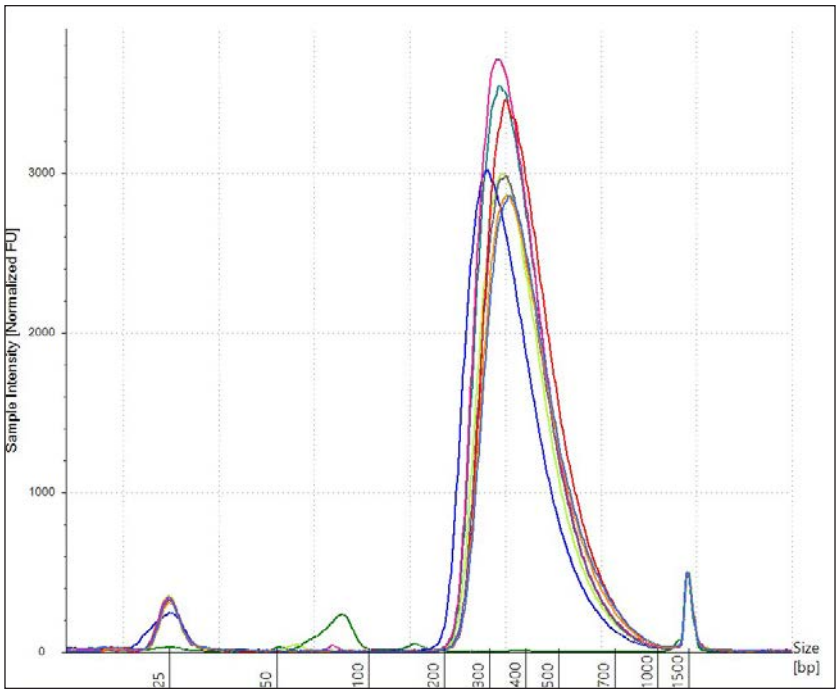

Figure 3. Agilent TapeStation trace results from libraries created on the Biomek NGeniuS system from 8 RNA samples (100 ng input mass) and one negative control using the NEBNext Ultra II Directional RNA Library Prep kit. Libraries have an average fragment size of 337 bp, averaged across all libraries created from these samples. Dark green trace is negative control.

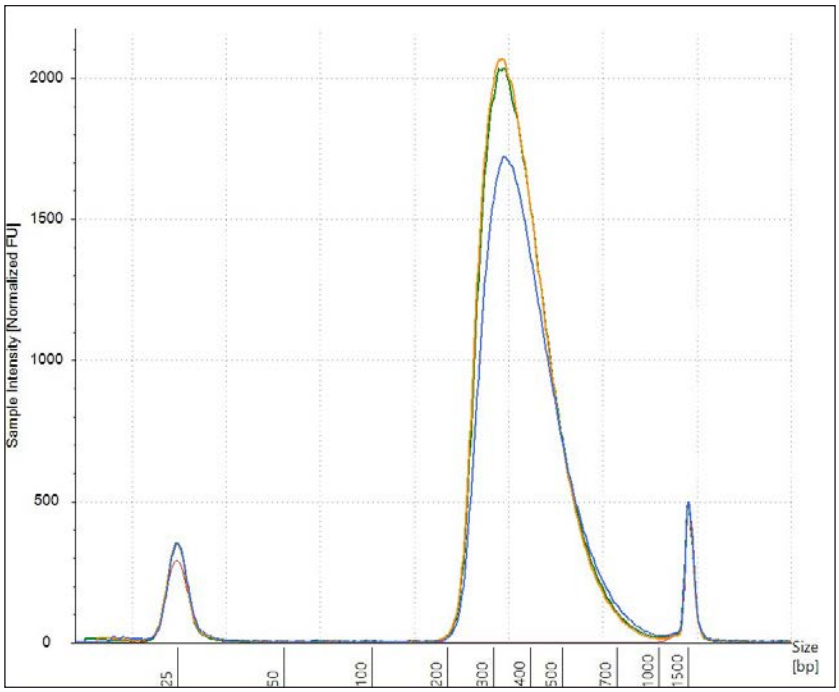

Figure 4. Agilent TapeStation trace results from libraries created on the Biomek NGeniuS system from 3 RNA samples (1000 ng input mass) and one negative control using the NEBNext Ultra II Directional RNA Library Prep kit. Libraries have an average fragment size of 327 bp, averaged across all libraries created from these samples. Red trace is negative control.

The combined NextSeq 1000 sequencing of 100 and 1000 ng library construction runs resulted in over 515M pass filter reads, generating a total of 109 Gbp of sequencing data with 94.5% of bases scored at Q30 or higher. Similarly, the sequencing of the 10 ng input mass run resulted in over 531M pass filter reads, generating a total of 113 Gbp of sequencing data and 94.6% of bases scored at Q30 or higher. Across runs, the average percent duplicates was less than 5%, average percent reads aligned was greater than 97%, and average percent stranded was greater than 99% **(Table 6)**.

Experiment	1	2	3
Sample count*	4	9	24
Library Prep Input Mass (ng)	1000	100	10
Library Size (bp)	327	337	321
Qubit Conc. (ng/µL)	7.35	10.41	7.40
Percent transcript (%) **	84.3	83.1	81.7
Percent unknown (%) **	5.9	6.0	5.8
Percent intron (%) **	6.4	7.5	8.6
Percent intergenic (%) **	1.9	2.1	2.2
Properly Paired (%) ***	98.9	97.8	86.4
rRNA (%) ***	0.3	1.4	12.5
Duplicates (%) ***	3.80	3.21	4.65
Median insert size (bp) ***	144	148	150
Average Reads Aligned (%) ****	97.66	97.90	97.82
Median CV ****	0.52	0.50	0.50
Average Stranded (%) ****	99.57	99.52	99.20

Table 6. Summary of result data, averaged for each experiment. * Each experiment includes one negative control, left out of sequencing data. ** Data from BaseSpace DRAGEN Analysis App. *** Data from DRAGEN RNA App. **** Data from RNA-Seq Alignment App.

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 **(Figures 2-4)** and fall within the recommended library size range of the NEBNext Ultra II Directional RNA Library Prep Kit. Across all samples processed, over 97.6% of reads were aligned to reference transcripts and less than 4.7% of reads were duplicates **(Table 6)**.

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. NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina® Instruction Manual, Version 4.0_4/21

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

2023-GBL-EN-104476-v1