APPLICATION NOTE

AUTOMATED SMALL RNA LIBRARY CONSTRUCTION USING THE NEBNext® SMALL RNA LIBRARY PREP KIT FOR ILLUMINA® ON THE BECKMAN COULTER Biomek® 4000 AUTOMATED LIQUID HANDLER

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Abstract

Small RNAs, constituting RNA species ranging from 17-35nt, play a role in numerous biological processes, including cell proliferation, metabolism, and cell death1. Next generation sequencing (NGS) technologies have allowed for a rapid expansion in small RNA research by allowing researchers to directly characterize small RNA populations from a variety of species. The New England Biolabs NEBNext® Small RNA Library Prep Kit for Illumina (Catalog Number E7300/7580) allows users to construct indexed Small RNA sequencing libraries with inputs ranging from 100ng to 1000ng of high quality total RNA. In this technical note we describe the automation of the NEBNext® Small RNA Library Prep Kit for Illumina on the Beckman Coulter Biomek 4000 automated liquid handler. The automation method includes an intuitive HTML-driven user interface for ease of operation as well as HTML-driven reagent calculator to show the operator the required reagents, their respective volumes, and their location on the instrument deck based on the user's input on number of samples and steps to be run. The Biomek 4000 NEBNext Small RNA method can prepare up to 24 indexed NEBNext® Small RNA libraries in approximately 9 hours. For higher throughput users, the NEBNext Small RNA Small RNA Library Prep Kit for Illumina has been implemented on the Biomek FX^P Dual Arm Multi-channel 96 and Span-8 automated liquid handler and can process up to 96 libraries in approximately 9 hours.

Method Overview and Hardware Description

The automation method described here was developed on the Biomek 4000 automated liquid handler. The deck configuration used is displayed in Figure 1, and a list of automated labware position (ALP) hardware is presented in Table 1. A static peltier unit ensures that enzyme master mixes are kept cool during the course of the method. For on-deck incubations, a Biometra T-robot integration is required, otherwise the method prompts the user to remove the plate from the deck and perform the incubation in an off-deck thermocycler. Filter tips are employed throughout the method to reduce the possibility of instrument or sample contamination. A list of automation consumables and user-supplied reagents can be found in Table 2 and Table 3 respectively.

Figure 1: Biomek 4000 NEBNext Small RNA automated deck layout.

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The automated method follows the NEBNext Small RNA Library Prep Kit for Illumina protocol closely and supports all official stopping points. 5ul of total RNA is added to a 96 well plate by the user, along with enzyme master mixes and other reagents as directed by the Reagent Calculator. The automation workflow is presented in Figure 2.

Figure 2: Biomek 4000 NEBNext Small RNA workflow. Library construction processes are highlighted in blue, library amplification is highlighted in red, and library size selection is highlighted in green.

The automated Biomek 4000 NEBNext Small RNA method features an HTML-driven User Interface (Figure 3) that provides the user with the ability to customize their workflow by offering a number of different options. These options include:

1. The ability to run the method as a complete workflow or as individual modules in accordance with the protocol.
2. The ability to specify the dilution factor of adaptors and primers for optimization of library preparation.
3. The flexibility to utilize either a 96 well plate or NEBNext index tubes in a Beckman Coulter 24 position tube rack for index primer deployment.
4. Functionality to deploy index primers to the sample plate automatically or using a file driven transfer for custom index primer deployment.
5. The choice of a number of post library construction size selection options, including AmpureXP based size selection of the final small RNA libraries.

Figure 3: Biomek 4000 NEBNext Small RNA User Interface.

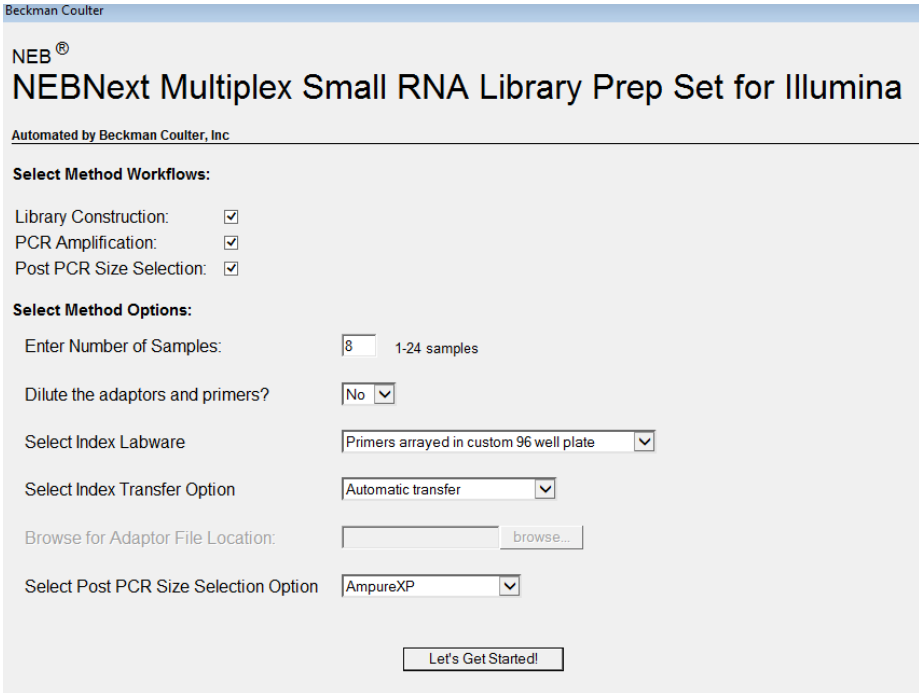
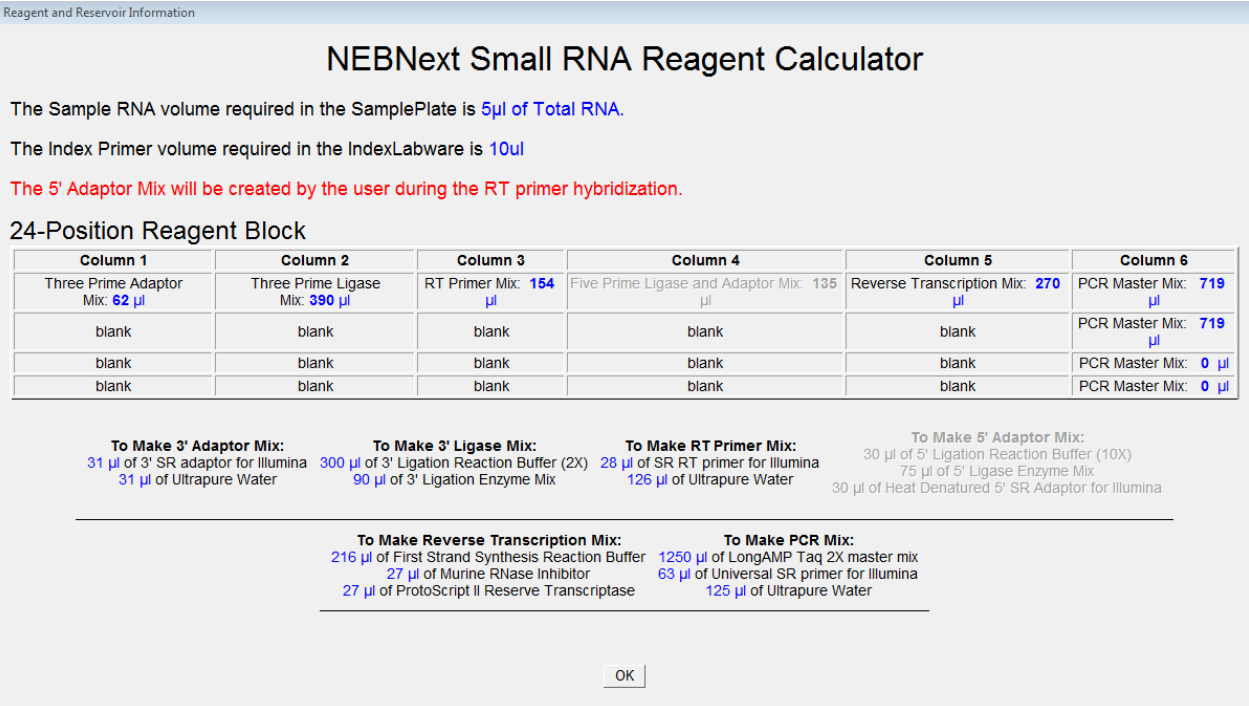


Figure 4: Biomek 4000 NEBNext Small RNA Reagent Calculator.



Based on these options, an HTML-driven Reagent Calculator will appear at various points during the method with instructions for how to make each enzyme master mix and where to locate it on the deck based on the number of samples being processed, adaptor and primer dilution factors, and the labware dead volume requirements. An image of the Reagent Calculator is presented in Figure 4.

Results

Three total RNA samples (Ambion Human Brain total RNA (Catalog Number AM7962), Ambion Human Colon total RNA (Catalog Number AM7986), Agilent Universal Human miRNA Reference RNA (Catalog Number 750700) were obtained from their respective suppliers and quantified using Quant-iT RiboGreen (Life Technologies). For each total RNA sample, a 500ng aliquot and a 100ng aliquot was resuspended in a final volume of 5ul and added to a 96 well PCR plate. The samples were then processed into NEBNext Small RNA libraries using the Biomek 4000 NEBNext Small automated method. A 1:3 adaptor/primer dilution was selected for these samples. Following 15 cycles of PCR amplification, the samples were purified using the Biomek 4000 NEBNext Small automated method and assayed using the Bioanalyzer 2100 (Agilent) with a D1000 chip. The libraries were size selected using the AmpureXP size selection option provided in the Biomek 4000 NEBNext Small automated method to remove fragments larger than 147bp. Following size selection, the libraries were again assayed using the Bioanalyzer 2100 (Agilent) with a D1000 chip. A representative trace showing the pre-size selection and post size selection libraries is shown in Figure 5.

Following size selection, the libraries were quantified using the Illumina Library Quantification kit (Kapa Biosystems) and sequenced on the Illumina MiSeq using a 36 cycle single read run. Approximately 16.2 million passed filter reads were generated; with over 97% of reads were identified following demultiplexing. Reads were processed on BaseSpace using the Illumina FASTQ ToolKit application to trim the reads. A histogram of reads after adaptor trimming is shown in Figure 6.

The trimmed reads were subsequently analyzed on BaseSpace using the Illumina Small RNA application. This application utilizes BowTie v0.12.8 to align the reads to the reference genome, followed by miRDeep v3.2 for novel miRNA prediction and DESeq2.0 v1.0.17 for differential expression analysis if desired. As shown in Figure 7, approximately 50% of reads mapped to either miRNA or other small RNAs. Abundant reads, which includes rRNA, 5SrDNA, mitochondrial DNA, polyA, polyC, and adaptors accounted for 27% to 41% depending on the library. As shown in Figure 8, the majority of the abundant reads consist of rRNA and 5SrDNA. Adaptor reads were 0.04% or less of abundant reads in all six libraries.

Figure 5: Agilent 500ng input NEBNext Small RNA library. Library prior to size selection is shown in blue. Post size selection library is shown in red.

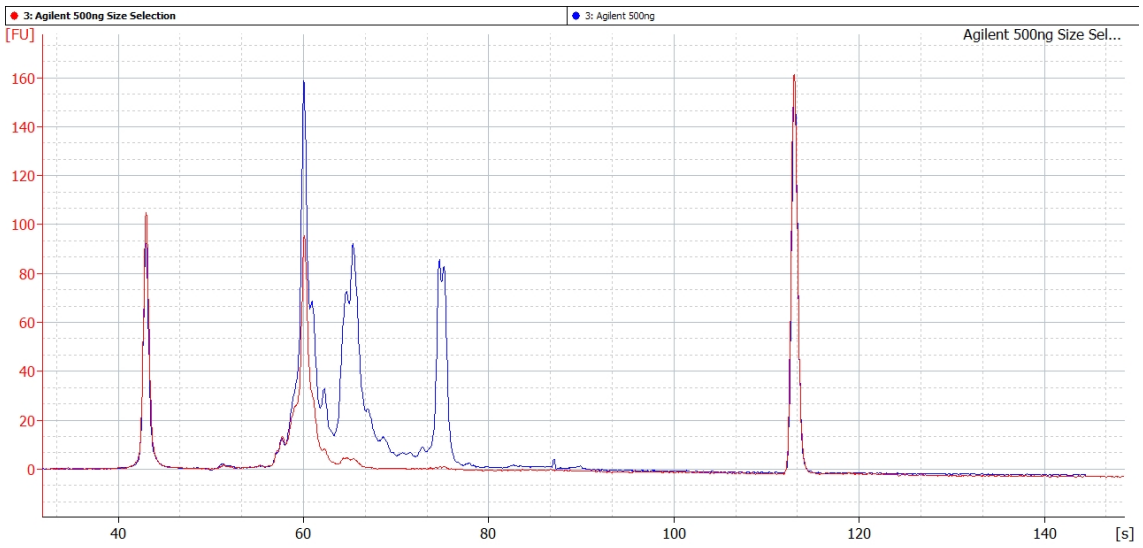
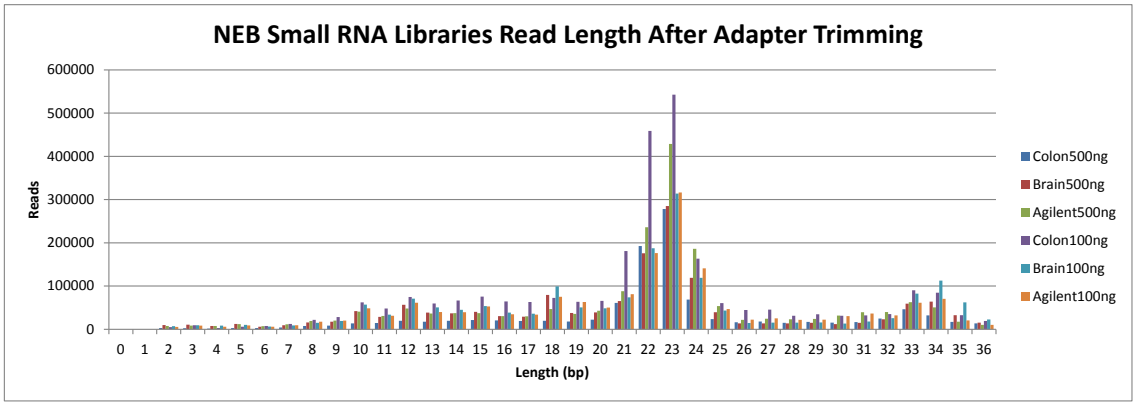


Figure 6: Read length histogram for all NEBNext Small RNA libraries following adaptor trimming.



Differential expression analysis performed on BaseSpace using the Small RNA application revealed broad agreement in expression within tissue types in spite of the use of different starting concentrations of RNA. As shown in Figure 9, brain and colon samples shows defined patterns of miRNA and isomiR expression while the Agilent miRNA control libraries show patterns of expression common to both brain and colon samples, which is expected given the makeup of cell lines the Agilent miRNA Reference RNA is derived from.

Figure 7: Percent read distribution for all NEBNext Small RNA libraries.

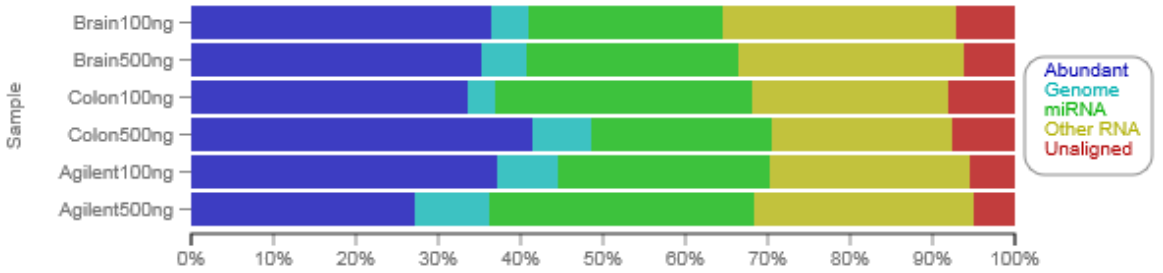


Figure 8: Percent abundant read distribution for all NEBNext Small RNA libraries.

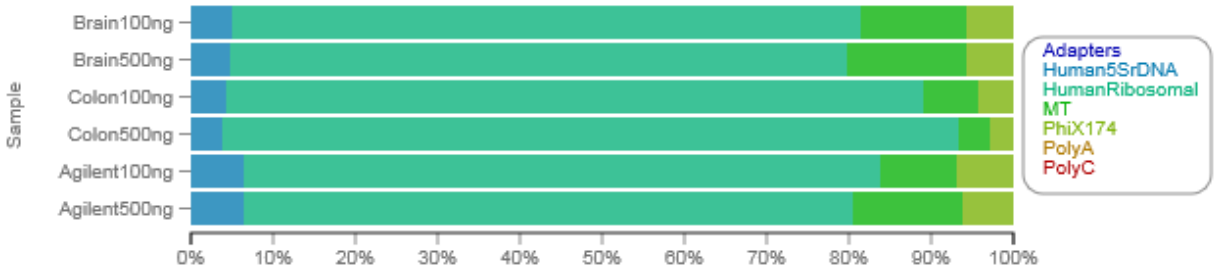
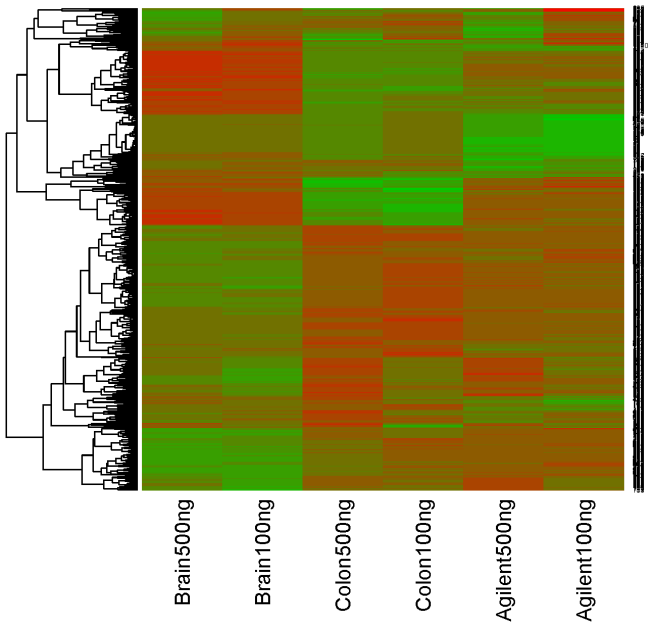


Figure 9: Heat map of miRNA and isomiR expression for the NEBNext Small RNA libraries. Green indicates high levels of expression, while red indicates low levels of expression.



Conclusion

In conclusion, the Biomek 4000 NEBNext Small RNA automation method provides a reliable solution for the creation of NEBNext Small RNA libraries that are highly enriched for miRNA species. The additional convenience of automated library size selection utilizing AmpureXP provides the user the ability to size select libraries without the need for polyacrylamide gels or purchasing additional equipment.

Table 1: Biomek 4000 hardware configuration

PART NUMBER	QUANTITY	MANUFACTURER	DESCRIPTION
B22640	1	Beckman Coulter, Inc.	Biomek 4000 Genomic Workstation
609119	1	Beckman Coulter, Inc.	Tool Rack, Five Liquid-Handling Tools
Contact Beckman Coulter, Inc.	1	Biometra	(Optional) Biometra T-Robot for On-Deck incubations

Table 2: Automation consumables

PART NUMBER	QUANTITY PER 24 SAMPLE RUN	MANUFACTURER	DESCRIPTION
B01124	1	Beckman	Biomek Span-8 P1000 Tips, Pre-sterile with Barrier
A21586	3	Beckman	Biomek P50 Tips, Pre-sterile with Barrier
717253	3	Beckman	Biomek AP96 P250 Tips, Pre-sterile with Barrier
372790	2	Beckman	Quarter Reservoir
534681	1	Beckman	Reservoir, Half
372795	1	Beckman	Frame for Reservoirs
373661	1	Beckman	24-Position Tube Rack with white inserts
A32782	1	Beckman	Agencourt® SPRIPlate® 96R - Ring Super Magnet Plate
A83054	1	Beckman	BCI Tube Block
89004-290	8	VWR	1.5mL SuperClear™ Screw Cap Microcentrifuge Tubes - Conical Bottom
3845-43001-0020	1	ritter Gmbh	2ml Ritter RipPlate
HSP-9641	3	Bio-Rad	Hard-Shell® Thin-Wall 96-Well Skirted PCR Plates
MSL-2022	1	Bio-Rad	Arched Auto-Sealing Lids*

Table 3: User Supplied Reagents

PART NUMBER	MANUFACTURER	DESCRIPTION
Not available	User Preferred	Elution Buffer – Nuclease free water, TE, 10mM Tris, pH 8.5
A63881	Beckman Coulter, Inc.	AmpureXP
AB00138-01000	American Bioanalytical	Ethanol
E7300(S/L) or E7580 (S/L)	New England Biolabs	NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (Set 1 or Set 2)

Software used in QC Testing

1. FastQ ToolKit. https://basespace.illumina.com/apps/1802801/FASTQ-Toolkit?preferredversion
2. Small RNA. https://basespace.illumina.com/apps/948948/Small-RNA?preferredversion

Citations

David P Bartel, MicroRNAs: Genomics, Biogenesis, Mechanism, and Function, Cell, Volume 116, Issue 2, 23 January 2004, Pages 281-297, ISSN 0092-8674, http://dx.doi.org/10.1016/S0092-8674(04)00045-5. (http://www.sciencedirect.com/science/article/pii/S0092867404000455)

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