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APPLICATION NOTE I No. 405

Automated library preparation using NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® on the ep*Motion*® 5075t

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Abstract

With the rapid growth of Next Generation Sequencing (NGS), generating robust libraries from precious samples is a key requirement for many NGS applications. Automation of library preparation not only minimizes the loss of samples, wasted reagents and sequencing delays, but also reduces inter-operator variability as well as errors in sample tracking. This application note describes successful automation of NEBNext® Ultra™ II Directional RNA Library Prep kit for Illumina® on the ep*Motion*® 5075t automated liquid handling system of Eppendorf.

High quality RNA-seq libraries were prepared from as little as 5 ng of total RNA input. The generated libraries had consistent insert size (peak size of approx. 300 bp) and were free of adapter dimers, primer dimers or over-amplification. Data generated using human RNA samples show high read mappability (> 95%), low percentage of ribosomal RNA (< 0.1%), and uniformity in coverage across the transcript length. Here we show that the automated workflow is robust and performs within the NEBNext® Ultra™ II Directional RNA Library Prep Kit specifications at a wide range of input amounts.

Introduction

RNA-seq is a powerful tool used to obtain in-depth information on expression profiling, gene annotation, and transcript discovery [1]. However, the low quality and quantities of source RNA can be a limitation. To address these challenges, NEB developed a second generation of strand-specific RNA library prep kits that delivers high quality libraries and enables use of lower input amounts with fewer PCR cycles. The NEBNext® Ultra™ II Directional RNA kit uses a broad range of input amounts (10 ng – 1 µg Total RNA for the polyA mRNA workflow and 5 ng – 1 µg Total RNA for the rRNA depletion workflow) and has a streamlined workflow amenable to automation.

Here, in partnership with New England Biolabs (NEB), we utilized our expertise from automating a wide variety of library preparation chemistries, to develop an automated workflow for the NEBNext® Ultra™ II Direction RNA Library Prep kit for Illumina® on the ep*Motion*® 5075t automated liquid handling system. We show that the automated workflow developed for the NEBNext® Ultra™ II Directional RNA on ep*Motion*® 5075t is able to accommodate a broad range of starting input amounts and sample throughputs.

Materials and Methods

ep*Motion* setup

The ep*Motion*® is a multi-purpose liquid handling workstation that is suitable for many laboratory procedures. The ep*Motion*® 5075t (Figure 1) and other models in the family are ideal walk-away companions for labs that demand high efficiency, accuracy and automated workflow. The on-deck Eppendorf Thermomixer® and the incorporated thermal module provide incubation capability needed for the NGS library preparation. Eight dispensing tools covering 0.2-1000 µL range are available in both single- and 8-channel formats to meet different throughput requirements. Additionally, a gripper can be added to transport plates around the worktable with ease.

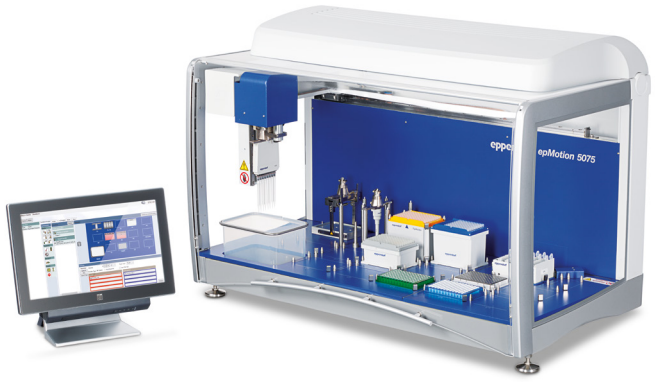

Figure 1: Eppendorf ep*Motion* 5075t automated liquid handling system

The user-friendly interface (Figure 2) of the ep*Motion*® and modular programing capabilities, gives the users flexibility, and also guides the operator through the run setup, including placement of the labware and required reagent volumes.

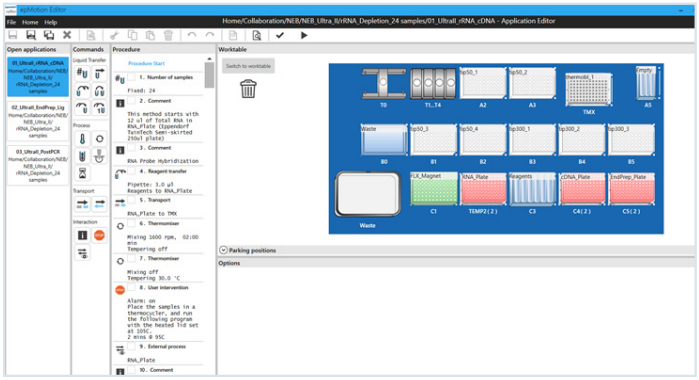

Figure 2: User Interface of Eppendorf's epBlue® software for ep*Motion*®.

For the automated liquid handling, an ep*Motion*® 5075t equipped with a thermal module, Gripper tool, Single-channel and Multichannel dispensing tools covering the volume range of 1 µL – 300 µL was used. Magnetic bead separation was performed using Magnum FLX® Magnet Adapter. The automated protocol is split into 3 sub-methods (Figure 3), each stopping at a safe point. All incubations were performed on-deck with the exception of RNA/Probe hybridization and the library amplification.

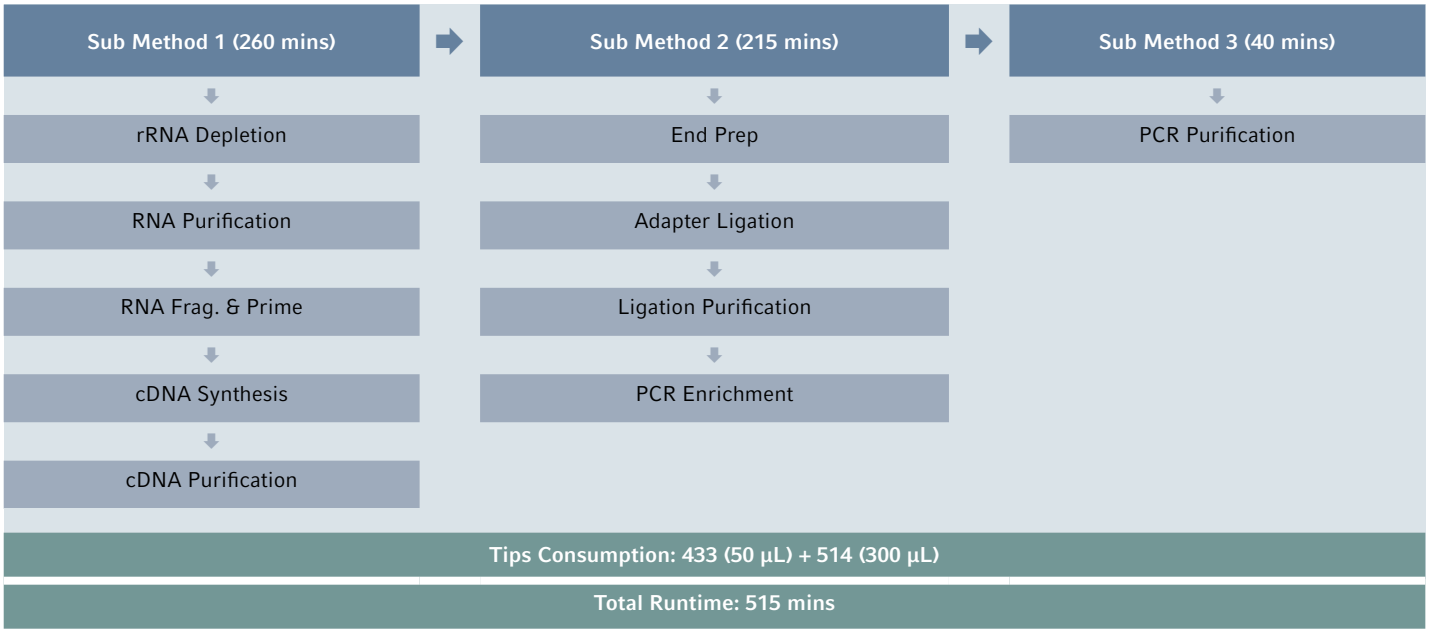

Figure 3: Workflow of the NEBNext® Ultra™ II Directional RNA Library Prep with rRNA Depletion protocol on ep*Motion*®. The steps are grouped into ep*Motion*® sub-methods. The methods are optimized for up to 24 samples. ep*Motion*® runtime and tip consumption estimate is for 24 samples.

Experimental design

RNA libraries were prepared using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® (E7760) and the NEBNext® Multiplex Oligos for Illumina® (Index primer Set 1, E7335), following the user guide recommendations. Different total RNA inputs (500, 100, 5 ng) of Universal Reference Human RNA (Agilent Technologies Inc.; Catalog No. 740000) and Mouse Kidney Total RNA was used to generate libraries. rRNA was depleted before library preparation using the NEBNext® rRNA Depletion Kit (Human/ Mouse/ Rat, E6130).

Three technical replicates were made for each input. All reaction setup and bead cleanups were performed in Eppendorf twin.tec® PCR Plates LoBind, semi-skirted [2]. RNA probe hybridization and PCR amplification was performed on Mastercycler® Pro S. The concentration of NEBNext® Adaptor and the number of PCR cycles used was as recommended in the manual, as shown in Table 1. For each library, the quality control (library size distribution and quantification) was performed using an Agilent® 4200 TapeStation® System. Libraries were diluted 10-fold and run on a HS tape. Sequencing was performed on an Illumina® NextSeq™ 500 System using paired-end mode (2X 76 bp).

Table 1: Experimental summary of ep*Motion* 5075t validation run.

RNA Input	Adapter Dilution	PCR Cycles
500 ng	5-fold	9
100 ng	25-fold	11
5 ng	200-fold	16

Results

Data Analysis

To demonstrate the robustness of the automated library preparation workflow, libraries were prepared for high (500ng), medium (100ng) and low (5ng) amounts of input RNA using two different source: human and mouse RNA. Library yields and size distribution was assessed using Agilent® 4200 TapeStation® system. Final libraries showed successful library prep with expected size distribution and mean peak at approximately 300 bp. The absence of adaptor and primer dimers indicate efficient library preparation and clean-up (Figure 4A). With minimal PCR cycles, sufficient yield of library was generated from all inputs (Figure 4B).

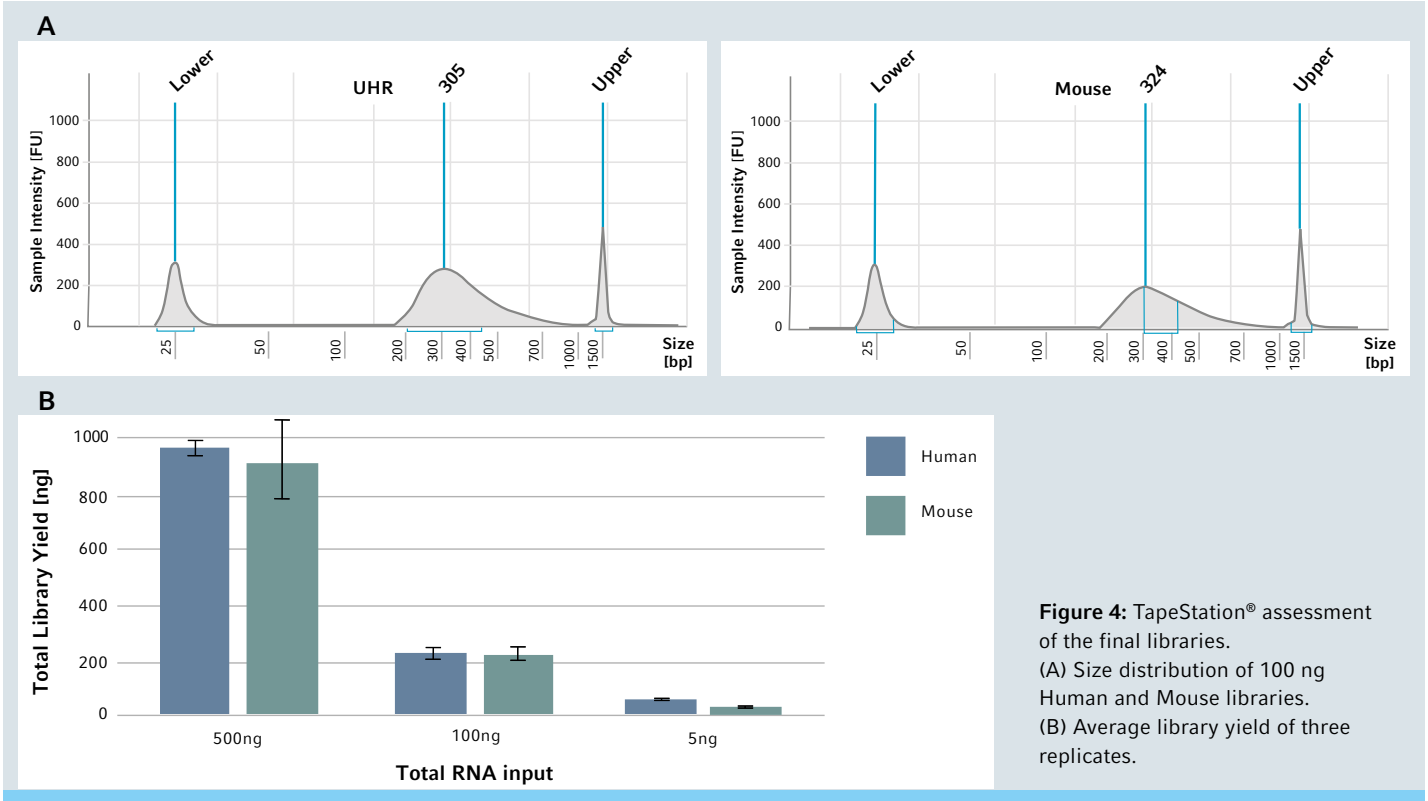

Figure 4: TapeStation® assessment of the final libraries.
(A) Size distribution of 100 ng Human and Mouse libraries.
(B) Average library yield of three replicates.

To further assess quality of the generated libraries, human RNA libraries were sequenced and data was analyzed using Picard tools. Reads were mapped to the hg19 reference genome using Hisat 2.0.3. All human libraries had high mapping rate and low percentage of adaptor dimer and chimeras; including the libraries made from low input (5 ng Total RNA) (Table 2). Mouse libraries also showed high percentage of mapping reads (95.5% for 500ng input, 94.7% for 100ng and 92.7% for 5ng inputs (Data not shown).

RNA Input	PF Reads Aligned	% PF Reads Aligned	% PF Reads Aligned in Pairs	% Filter (too short, adapter, dimer etc.)	% Chimera
500 ng	19,176,638	95.93	97.60	0.04	0.58
	19,172,671	95.90	97.57	0.04	0.60
	19,102,419	95.56	97.40	0.05	0.58
100 ng	19,153,108	95.80	97.45	0.04	0.63
	19,172,991	95.90	97.53	0.03	0.63
	19,095,498	95.52	97.34	0.04	0.63
5 ng	19,012,990	95.18	97.44	0.12	0.55
	18,984,015	95.02	97.46	0.11	0.56
	19,014,807	95.23	97.54	0.17	0.54

Table 2: Picard Alignment Summary metric for the human RNA libraries. Average of N=3.
Pf Reads Aligned: *Reads mapped to hg19 reference genome. All samples were down-sample to approx. 20 Million reads.*

% Pf Reads Aligned: *The percentage of Pf reads mapped to hg19 reference genome.*

% Pf Reads Aligned in Pairs: *The percentage of Pf reads whose mate pair was also aligned to the reference.*

% Filter: *The percentage of reads mapped to adaptor sequences or shorter than 30bp.*

% Chimeras: *The percentage of reads that map outside of a maximum insert size.*

Picard's tool was also used to calculate the distribution of mapped reads. As expected for ribosomal-depleted RNA libraries, both coding (exonic) and non-coding (intronic and intergenic) RNA reads are retained in the libraries (Figure 5). Ribosomal RNA was efficiently depleted from human RNA libraries (rRNA mapped reads is < 0.1% for all inputs).

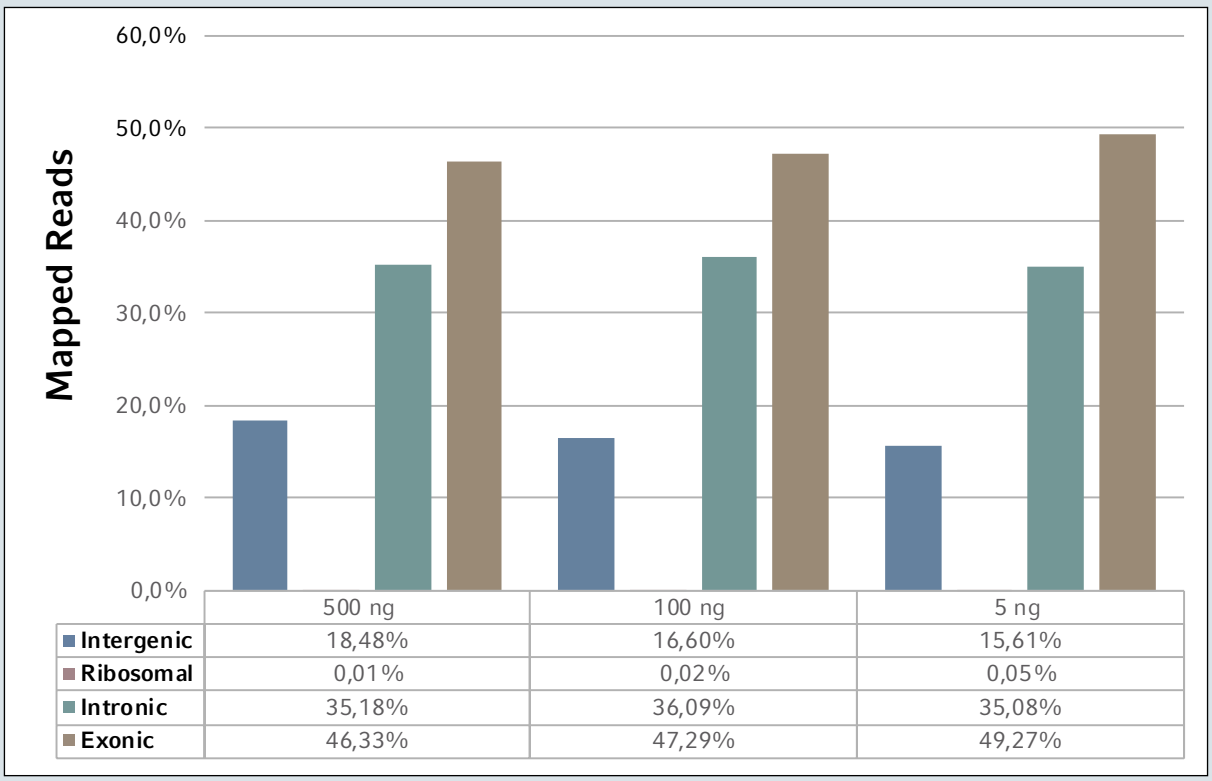

Figure 5: Average distribution of mapped reads for Human RNA libraries calculated with Picard. Less than 0.1% of total reads mapped to the rRNA region.

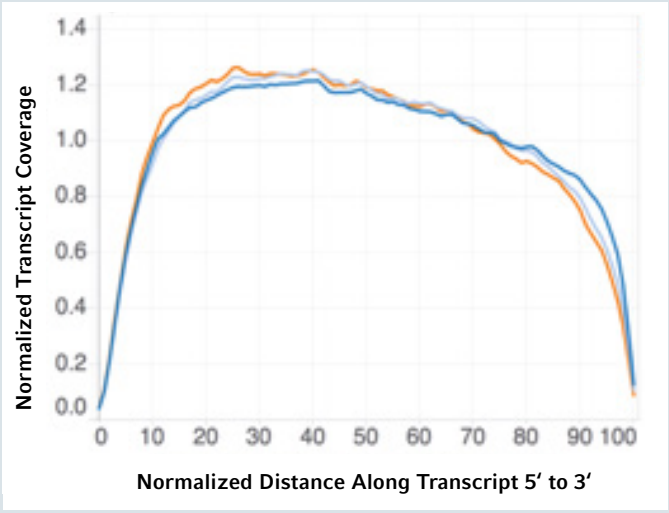

Figure 6: : Normalized RNA-seq coverage (5′ – to – 3′ sequence coverage) for 500 ng, 100 ng and 5 ng inputs. Average transcript coverage of all replicates shows.

Another measure of quality, associated with RNA library construction, is the evaluation of potential biases in transcript representation (coverage at 5′- and 3′- end) (3). The continuity of transcript coverage, i.e. coverage variation along the 5′ and 3′ ends of each transcript, was assessed among all libraries. The global view of the 5′ to 3′ coverage of the RefSeq (4) transcripts reveals uniformity across the transcript length (Figure 6).

Conclusion

NGS library construction is a critical step in sample preparation for sequencing on Illumina platforms. The increased demand for high-quality NGS libraries in high-throughput laboratories has necessitated the development of robust, automated methods for library preparation. New England Biolabs has partnered with Eppendorf to automate the NEBNext Ultra II Directional RNA Library Prep Kit on the Eppendorf ep*Motion* 5075t using the NEBNext rRNA Depletion Kit. The experimental data shows that the automated method performs within the NEBNext Ultra II Directional RNA Library Prep Kit specifications at a wide range of input amounts from 5 ng–500 ng. The highly flexible and modular automated solution for NGS library preparation will allow laboratories to easily scale up experiments without sacrificing quality in the process.

Literature

[1] Zhao S, et al. Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cells. PLoS One. 2014;16;9(1): e78644
[2 Hanae A. Henke, Björn Rotter. Eppendorf twin.tec® PCR Plates 96 LoBind Increase Yield of Transcript Species and Number of Reads of NGS Libraries. Eppendorf Application Note 375
[3] Levin, J. Z. et al. Comparative analysis of RNA sequencing methods for degraded or low-input samples. Nature Methods. 2010;7(9): 709-715
[4] O'Leary, N. A., et al. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res. 2016;44 (D1), 733-745.

Ordering information

Description	Order No. international	Order No. North America
ep*Motion*® 5075t NGS Solution	5075 000.962	5075000965
Thermoadapter PCR 96 (1x)	5075 787.008	960002199
Reservoir Rack 3 (1x)	5075 754.070	
Reservoir Rack Module TC, 4 × 0.5/1.5/2.0 mL (2x)	5075 799.081	960002620
Reservoir Rack Module TC, 8 × PCR tubes 0.2 mL (2x)	5075 799.049	960002601
Eppendorf twin.tec® PCR Plates LoBind, semi-skirted	0030 129.504	0030129504
Eppendorf Safe-Lock Tubes, 1.5 mL	0030 120.086	022363204
Eppendorf Fast PCR Tube Strips, 0.1 mL	0030 124.820	0030124910

New England Biolabs		
NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®		E7760
NEBNext® Multiplex Oligos for Illumina®		E7335*
NEBNext® rRNA Depletion Kit		E6130

*The volumes provided in the NEBNext® Ultra™ II Directional RNA library prep (E7760S) (24-reaction kit) are sufficient to prepare 24 libraries in one single ep*Motion*® run. However, the volumes could become limited if the kit is used in multiple runs. The volumes provided in the NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1) (E7335S) (24-reaction kit) become limited in a single 24-reaction run due to the need to transfer the primer solution from the original tube to strip tubes before dispensing them to the sample. To address this, we would recommend the use of the NEBNext® Multiplex Oligos for Illumina® (96 Index Primers) E6609S or the NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs) E6440S as in these products the primers are provided in 96-well plates.

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