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APPLICATION NOTE No. 493

Automated RNA Library Preparation for Whole Transcriptome Sequencing Using Watchmaker Genomics' RNA Library Prep Kit with Polaris™ Depletion on the epMotion® 5075t workstation

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Abstract

Library preparation plays a crucial role in generating quality sequencing data, and with increased interest in the transcriptome comes a demand for higher throughput methods for RNA library preparation. Automation can help to meet the increased throughput demands for Whole Transcriptome Sequencing (WTS) while robust and sensitive RNA library preparation workflows can ensure sequencing data integrity. Here we describe an optimized automated workflow for RNA library prep which includes improved scalability, sensitivity and turnaround times. Eppendorf has partnered with Watchmaker Genomics to automate the RNA Library Prep Kit with Polaris Depletion on the epMotion 5075t offering a streamlined workflow to generate high quality sequencing libraries from RNA inputs compatible with low and degraded sample types.

Introduction

The power of Whole Transcriptome Sequencing is unrivaled in gaining a comprehensive understanding of complex biological systems, especially in response to environmental stimuli. Quantifying changes in RNA transcription related to disease or altered states can be a powerful tool allowing researchers to unravel biological mechanisms, detect and monitor diseases and validate biomarkers and drug responses. Because of this, having a highly sensitive and robust sample preparation workflow is vital. In addition, sample RNA is not always abundant and being able to work with small amounts of RNA allows for the processing of scarce samples that were previously inaccessible.

Watchmaker's RNA Library Prep Kit with Polaris Depletion provides high quality RNA library preparation even with challenging conditions, such as degraded or low input sample types. The streamlined Polaris Depletion module depletes highly abundant rRNA and globin transcripts in human, mouse, and rat samples, providing improved coverage of biologically interesting transcripts, including long non-coding RNAs. The automation-friendly workflow reduces total turnaround time, hands-on time, and consumable use through a reduction in bead purification and reaction steps. A novel, engineered reverse transcriptase improves the conversion of RNA to cDNA, enabling high-quality performance with 1 ng to 1000 ng of total RNA, as well as FFPE-derived RNA. Additionally, the kit has impressive speed generating high-complexity libraries in as little as 4.5 hours, including depletion.

Automation is key in enabling a scalable solution that leverages a highly efficient workflow to maintain sample and data quality. When processing larger numbers of samples, automated liquid handlers, such as the Eppendorf epMotion 5075t, can help to ensure proper pipetting technique for high accuracy and precision. Eppendorf and Watchmaker Genomics have partnered to develop an automated workflow for the RNA Library Prep Kit with Polaris Depletion on the epMotion 5075t.

In cooperation with Watchmaker Genomics

WATCHMAKER GENOMICS

Automated Workflow Overview

The RNA Library Prep Kit with Polaris Depletion workflow on the epMotion 5075t has been optimized to allow RNA library preparation of up to 96 samples simultaneously. The epBlue software is user friendly, allowing anyone from a novice to an experienced epMotion user to generate high-quality RNA libraries. Before any run starts, the epMotion uses its onboard optical sensor, scanning the deck to ensure proper set up of the workspace. The epMotion performs all liquid transfer steps on-deck maximizing user's time away from the machine. The automated workflow is split into 4 separate sub-methods (Figure 1). The user is provided with guidance for setting up the deck, depending on the sub-method selected to run (Figure 2 A – D).

Figure 1: 4 separate sub-methods are included to run Watchmaker's RNA Library Prep Kit with Polaris Depletion on the epMotion 5075t.

*Safe stopping point, where samples can be covered and placed at 4 °C for less than 24 hours or -20°C for up to 4 weeks in concordance with Watchmaker's User Guide.

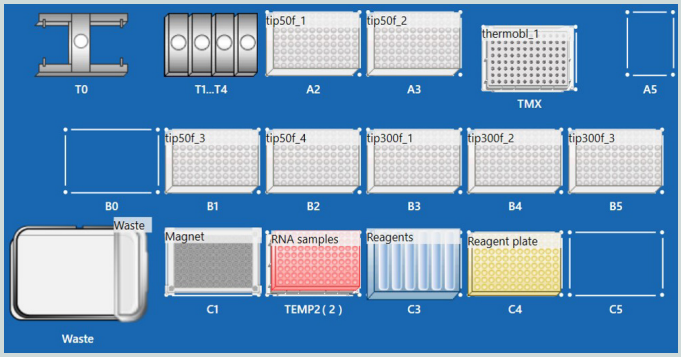

Figure 2A: Sub-method 1 deck layout, depletion through probe digestion.

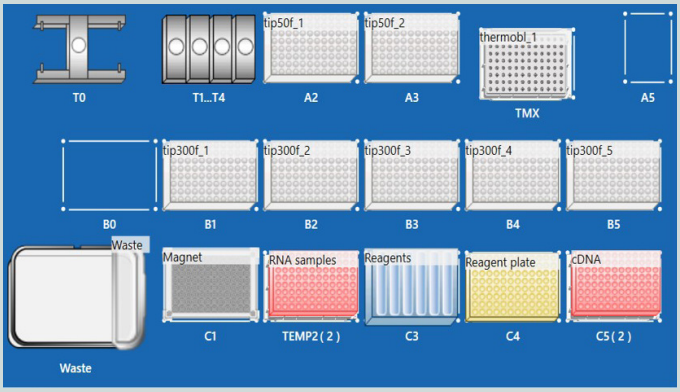

Figure 2B: Sub-method 2 deck layout, post-depletion cleanup through second strand synthesis and a-tailing.

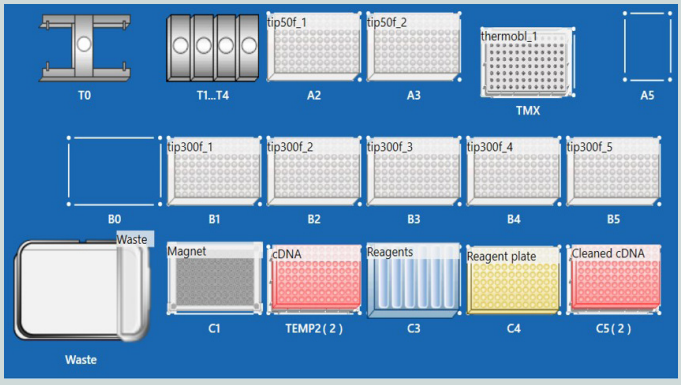

Figure 2C: Sub-method 3 deck layout, adapter ligation through library amplification.

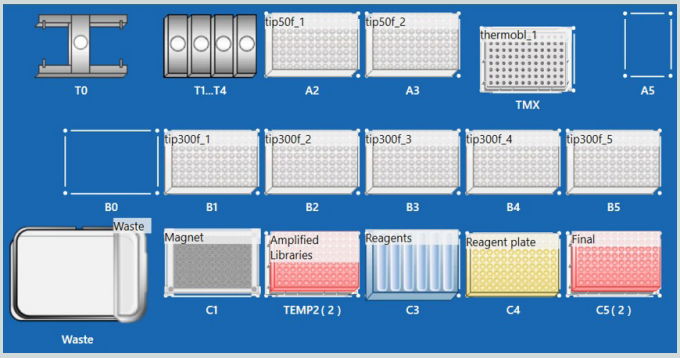

Figure 2D: Sub-method 4 deck layout, post-amplification cleanup.

Experimental Design, Materials, and Methods

Libraries were generated following the automated epMotion protocol for Watchmaker's RNA Library Prep Kit with Polaris Depletion (7BK0002-096). For all experiments included, 100 ng inputs were used. Fragmentation was performed following Watchmaker's guidance for high quality RNA, 85°C for 5 min, with a total of 12 PCR cycles. IDT xGen Stubby Adapters were used at Ligation with the xGen UDI Index Primers at PCR. All liquid handling steps were performed by the epMotion 5075t equipped with a thermal module, thermal mixer module, gripper tool, and signal and multi-channel tools (50 µl and 300 µl). Magnetic separations were performed using the Alpaqua Magnum FLX magnet. Incubations were performed in an off-deck thermal cycler following Watchmaker's User Guide.

For the first experiment, 8 samples of RNA isolated from human primary cells were prepared manually and then run in quadruplicate through the automated workflow for a total of 32 automated samples. This experiment was used to evaluate the initial performance of the automated workflow in comparison to manual processing when using high quality samples.

A second experiment was designed to assess the capabilities of the automated workflow at higher throughputs, this time including sequencing performance on real samples rather than high quality controls. Here a total of 71 RNA samples isolated from mouse spleen tissue were included on a single automated run. All samples included were assessed for their quality prior to starting library prep on the Agilent Fragment Analyzer using the DNF-471 RNA Kit and had RIN scores of >7. Final library yields were determined using the Thermo Fisher Scientific Qubit® Fluorometer with the dsDNA HS Assay Kit. Final library sizes were determined using the Agilent 2100 Bioanalyzer and the DNA 1000 Kit and Reagents. Following library preparation, all samples were sequenced on the Illumina Novaseq 6000 using an S2 Flow Cell and 2 x 100 bp reads.

Results and Discussion

For the first experiment, final library yield and sizes were comparable across both automated and manual preps (Figures 3, 4 and 5). Automated final library yield CVs within technical replicates were all below 15% (outliers removed). Automated final library size CVs within technical replicates were all below 5%. The observed results showed comparable performance between automated and manual methods allowing for the confident progression onto the second experiment.

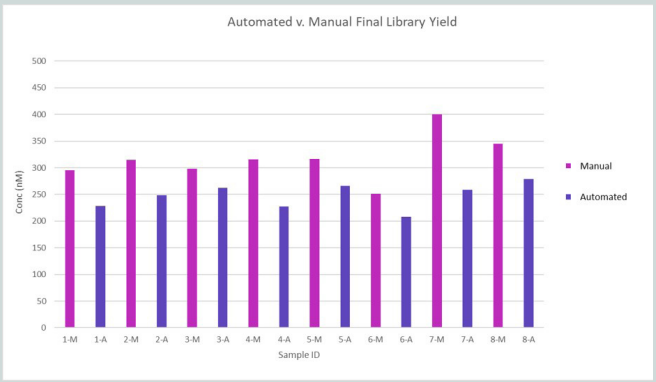

Figure 3: Automated final library yields were compared to manual final library yields across 8 samples. Observed differences were minimal with few outliers.

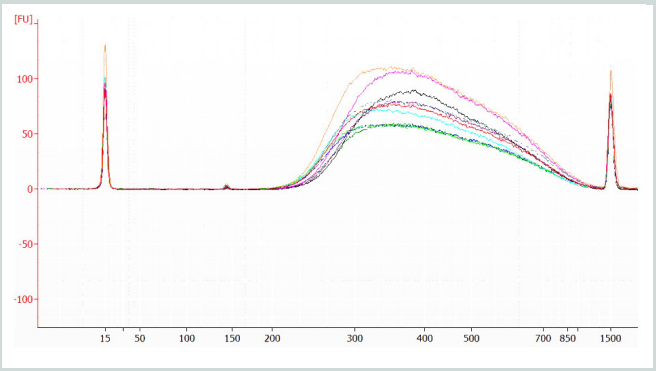

Figure 4: Final library traces showed comparable sizes within all automated samples.

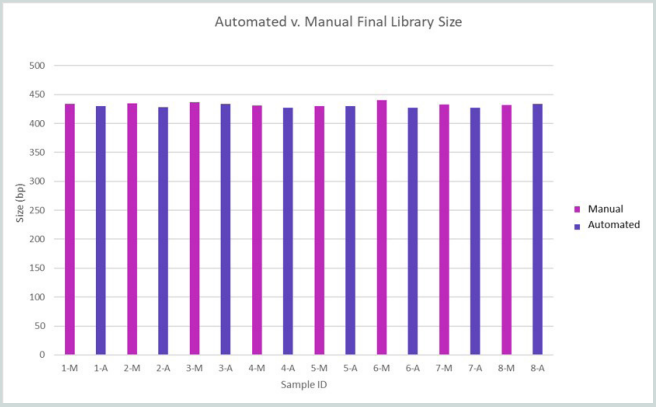

Figure 5: Final library traces showed comparable sizes between manual and automated libraries.

Results and Discussion (cont.)

For the second experiment, including 71 mouse spleen tissue samples processed on a single automated run, the final library yields showed that all samples produced enough yield to proceed with sequencing (Figure 6). Variability in final library yield across unique samples is to be expected, and more importantly all samples ran successfully to produce sequenceable libraries with no sample failures. Final library sizes showed the expected average sizes of ~430 bp (Figure 7). Sequencing summary metrics showed on average 93% total pass filter reads aligned, 86% uniquely mapped paired reads per sample and 96% strandedness. Very low residual rRNA (0.00312%) was observed across all samples, and the number of genes detected per sample was upwards of 36,000 (Figures 8 A - E). Final sequencing results demonstrate that the automated workflow can produce high quality sequencing data when processing large numbers of RNA samples in a single run.

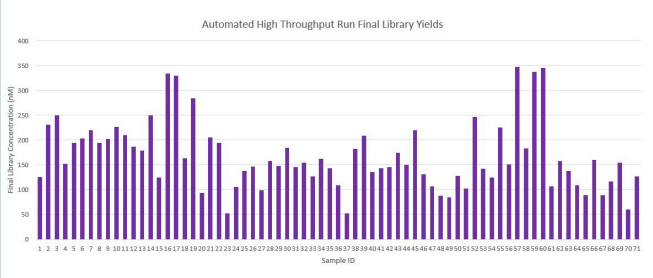

Figure 6: Automated final library yields showed that all final libraries had enough yield to proceed to sequencing with no sample failures.

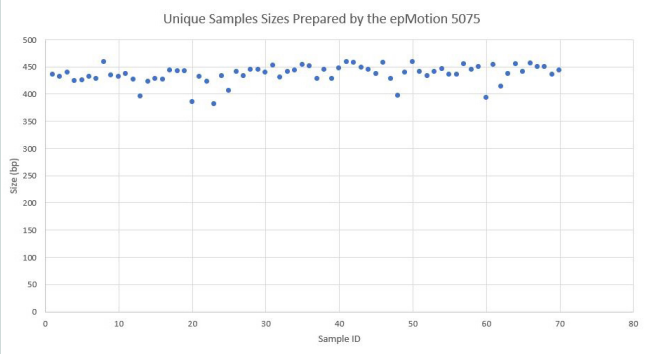

Figure 7: Automated final library sizes showed consistency across all individual samples.

Avg % Uniquely Mapped Paired Reads/Sample	Avg % Mapping to rRNA	Avg % Correct Strand	Avg % Fragment Duplicates (≥+1)
86%	.00312%	96%	23.6209694%

Mean Insert Size	Avg. Number of Genes Detected/Sample	% Total Pass Filter Reads Aligned
227	36,600	93%

Figure 8A: Sequencing summary metrics for high throughput automated run.

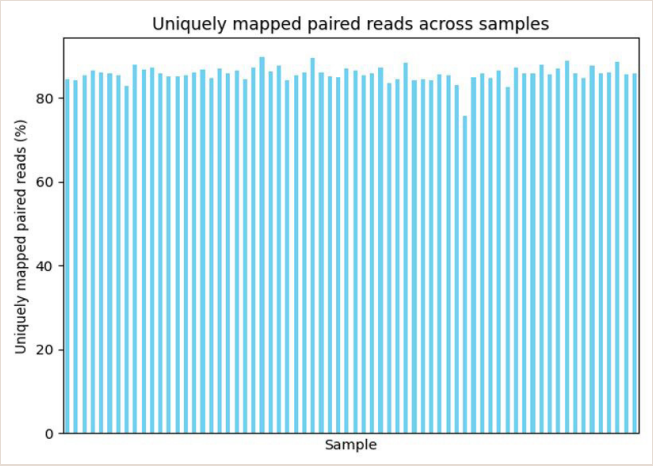

Figure 8B: Uniquely mapped paired reads across samples.

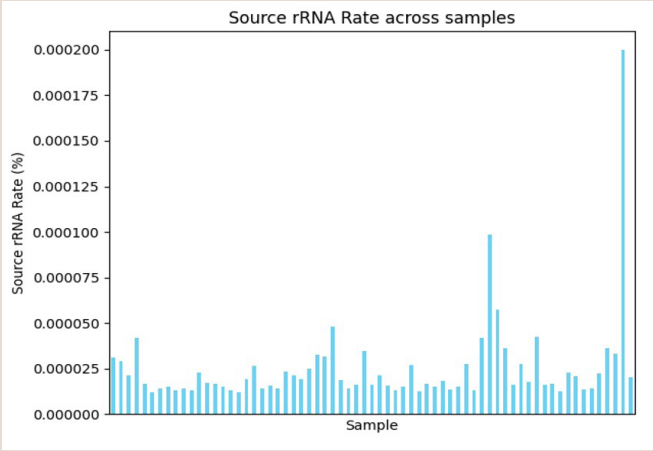

Figure 8C: Source rRNA rate across samples.

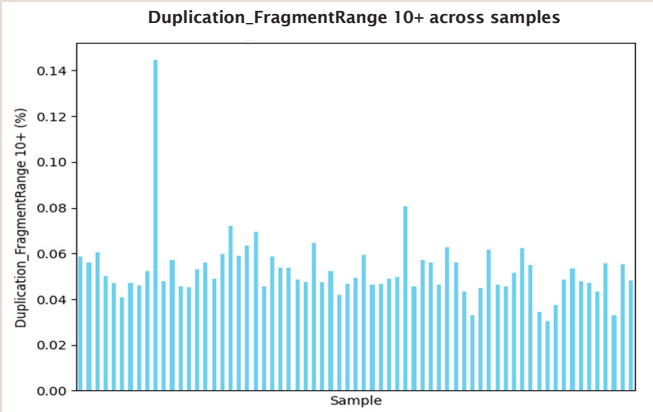

Figure 8D: Duplication Fragment Range (at least 10+ per sample).

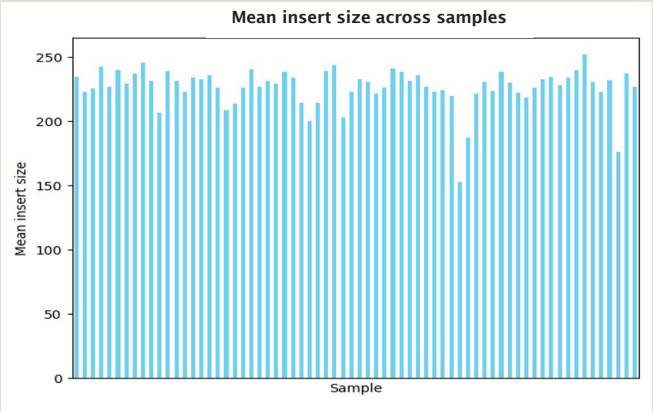

Figure 8E: Mean insert size across samples.

Conclusion

Generating high-quality, accurate, and repeatable libraries are essential to meet the growing demand for WTS data. Here we demonstrate a novel scalable workflow for RNA library prep using Watchmaker's RNA Library Prep Kit with Polaris Depletion automated on the Eppendorf epMotion 5075t. The streamlined automated process leverages a high performing kit alongside reliable automation, which generates quality sequencing data from a range of RNA inputs while freeing up hands-on time in the lab.

For more information on Watchmaker Genomics, please visit: watchmakergenomics.com

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