Evaluating a rapid automated RNA-Seq workflow for ultra-low input sample types

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Introduction

Next-generation sequencing (NGS) continues to revolutionize genomics, with ongoing advancements driving greater sensitivity, accuracy, and scalability across diverse applications. With RNA-Seq technology being more broadly adopted, there's a growing demand for streamlined workflows capable of processing clinically relevant sample types. This study evaluates the robustness of the Watchmaker RNA Library Prep Kit with Polaris™ Depletion when automated on the Hamilton Microlab® NGS STAR MOA using ultra-low input samples.

Simple and Rapid RNA Library Prep

The Watchmaker RNA Library Prep with Polaris™ Depletion can rapidly prepare stranded, whole transcriptome sequencing libraries. The highly streamlined Polaris Depletion module depletes highly abundant ribosomal RNA (rRNA) and globin transcripts in human, mouse, and rat samples, providing improved coverage of biologically interesting transcripts, including long non-coding RNAs. 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, for both high-quality and FFPE-derived RNA (Figure 1).

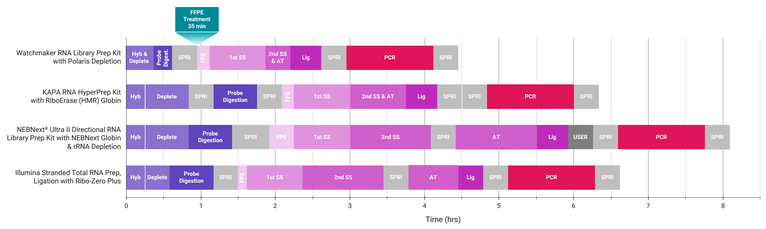

Figure 1. Improve automatability and reduce turnaround time. The Watchmaker solution combines and shortens enzymatic steps and has fewer bead purifications in comparison to commercially available kits, resulting in a highly automatable workflow with significantly reduced hands-on time (up to one hour per 96 plate) and consumable use (up to 1,000 pipette tips per 96 plate).

Dynamic Automated Method

The Hamilton Microlab® NGS STAR MOA is an automated liquid handler with a 96 Multiprobe Head (MPH), 8-independent channels, and a On-Deck Thermal Cycler (ODTC) allowing full walk-away sample processing. The Watchmaker RNA Library Prep Kit with Polaris Depletion automated script enables users to process 1 – 96 samples in a single run and can generate 96 high-quality sequencing ready libraries in under 7 hours. The method uses dynamic run-time options supporting multiple workflows at runtime (Figure 2).

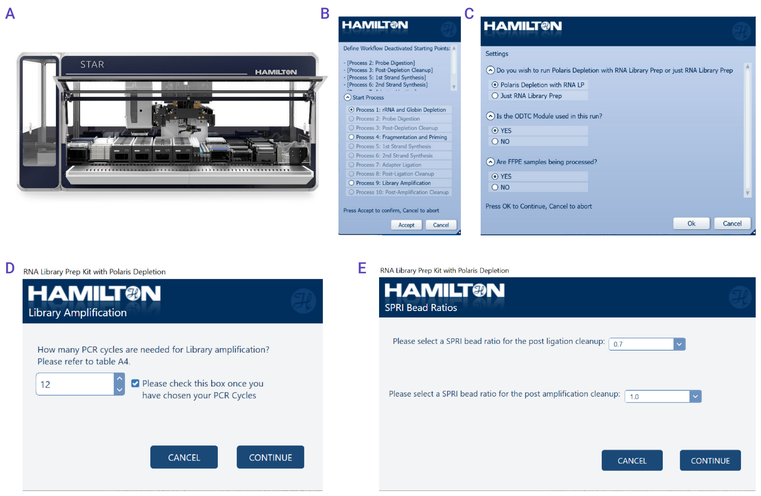

Figure 2. Dynamic user options. The **(A)** Hamilton Microlab® NGS STAR MOA enables flexibility and speed utilizing both its MPH and 8-independent channels. The Watchmaker RNA Library Prep with Polaris Depletion script includes **(B)** dynamic runtime user options for safe start/stop points, **(C)** run conditions, **(D)** PCR cycles, **(E)** SPRI bead cleanup ratios, and more – supporting multiple workflows within a single automated method.

Establishing Method Performance

The performance of the automated method was assessed using two separate runs. In the first run, 48 replicates of high-quality RNA (Agilent Human Reference RNA) were diluted to a total concentration of 50 ng per replicate alongside 48 No Template Controls (NTCs) in a checkerboard pattern, observing for the absence of cross-contamination and plate effects. Additionally, manual libraries were prepared using the same input amount and library prep conditions to compare against the automated replicates. The first run generated libraries with consistent concentration (<10% CV) and size (<1% CV), both across columns and rows, while being comparable to manual libraries (Figure 3). All NTCs were free of cross-contamination (data not shown).

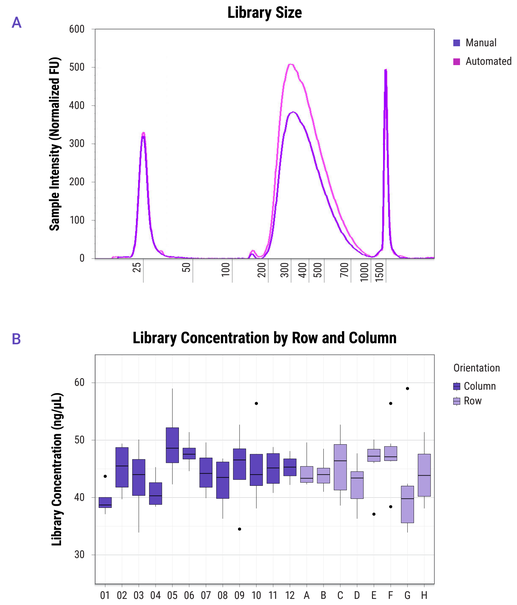

Figure 3. Final library QC. (A) Final library size was measured using the TapeStation 4200 on a High Sensitivity ScreenTape concentration. **(B)** Final library concentration was measured on the Qubit 4 Fluorometer using the 1X dsDNA High Sensitivity Assay.

Evaluating Low-Input Samples

The second run involved a full plate of ultra-low high-quality RNA replicates, with 5 NTCs included to assess the method's performance at the kit's lower input limit. In parallel, manual and automated library preparations were conducted, followed by sequencing of a subset of both on an Illumina NextSeq 2000 using a P3 Flow Cell and 2 x 150 bp paired-end reads.

The following experimental conditions were used for the second automated run:

- Sample Type: **1 ng Agilent Human Reference RNA**
- Fragmentation: **85°C for 5 minutes**
- Adapter Type: **xGen™ Stubby Adapter-UDI Primers**
- Adapter Concentration: **0.2 µM**
- PCR Cycles: **18**
- Post-Ligation SPRI Cleanup Ratio: **0.7X**
- Post-Amplification SPRI Cleanup Ratio: **1.0X**

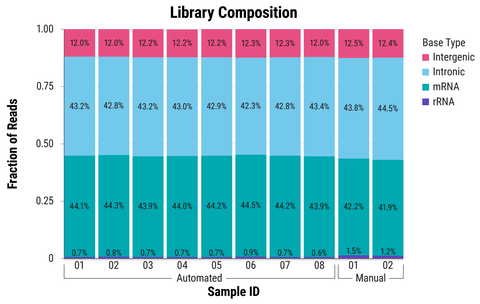

Figure 4. Base burndown comparison between preps. Comparison of base type distribution in RNA-seq libraries between automated and manual library preparation showing comparable base distribution of intergenic, intronic, mRNA, and rRNA bases. All sequencing data was down sampled to 20 million reads.

Consistent, High Library Complexity

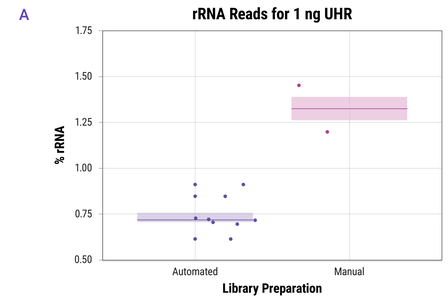

Figure 5. Assessment of rRNA contamination and unique gene detection. No significant differences were observed for **(A)** residual rRNA and **(B)** unique gene count between automated and manual library preparation methods.

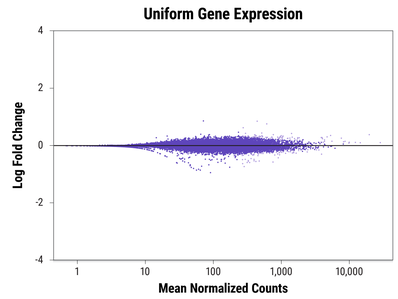

Figure 6. Differential expression of genes. Consistent gene expression profiles across low and highly expressed transcripts when comparing automated and manual library preparation methods.

These data underscore the reliable performance of the automated library preparation method for ultra-low RNA inputs when compared to traditional manual techniques. Both approaches yield comparable results for key sequencing metrics such as base burndown (Figure 4), rRNA contamination (Figure 5A), and unique gene detection (Figure 5B), demonstrating that the automation does not compromise the integrity of the libraries. These metrics are essential in ensuring that sequencing data is of high quality, free from bias, and accurately reflects the transcriptome of interest. Notably, the consistency in gene expression profiles between both methods — even at the lower RNA input limits of the library prep kit — highlights the robustness of the automated protocol (Figure 6) which is particularly important for researchers working with limited or degraded sample types, confirming that the automated system can generate reliable and reproducible data without sacrificing performance.

Conclusions

The Watchmaker RNA Library Prep Kit with Polaris™ Depletion on the Hamilton Microlab® NGS STAR MOA demonstrates the following:

- Supports multiple workflow options within a single method, enabling flexibility for a wide range of RNA sequencing applications (Figures 1 and 2)
- Reduces variability of library preparation, improves efficiency, and minimizes operator error, making it valuable for high-throughput and time-sensitive applications (Figure 3)
- Generates high-quality, sequenceable libraries at ultra-low inputs as demonstrated by comparable QC and sequencing metrics between automated and manual preparations (Figures 4, 5, and 6)