

Watchmaker RNA Library Prep Kit with Polaris® Depletion Automated on Hamilton Microlab® NGS STAR

NGS, Whole Transcriptome Sequencing,
and Automated Ultra-Low Input Total RNA Library Prep

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Introduction

Next-Generation Sequencing (NGS) continues to revolutionize genomics, enabling groundbreaking advances in research and diagnostics by offering unparalleled sensitivity, accuracy, and scalability. As RNA-Seq technology becomes more broadly adopted, there is an increasing demand for streamlined workflows that can handle diverse, clinically relevant sample types with efficiency and precision.

Leveraging automation for RNA-Seq library prep can offer enhanced reproducibility, reduce hands-on time, and minimize user error. When coupled with a high performance workflow, it enables high-throughput applications that maximize sequencing quality while minimizing cost.

This technical note evaluates the robustness and performance of the Watchmaker RNA Library Prep Kit with Polaris Depletion automated on the Hamilton Microlab® NGS STAR standard configurations. This automated Whole Transcriptome Sequencing (WTS) workflow offers both flexibility and usability, making it easily accessible for high-throughput research and clinical settings.

Methods & Features

The Watchmaker RNA Library Prep Kit with Polaris Depletion can rapidly prepare stranded, whole transcriptome sequencing libraries. The streamlined Polaris Depletion module depletes highly abundant ribosomal RNA (rRNA) and globin transcripts in human, mouse, and rat samples, providing improved coverage of biologically relevant transcripts, including long non-coding RNAs. A novel, engineered reverse transcriptase improves the conversion of RNA to cDNA, enabling high-quality performance across a range of inputs from 1 ng to 1000 ng of total RNA, including challenging sample types such as degraded and FFPE-derived RNA (Figure 1).

The Hamilton Microlab® NGS STAR system offers two standard configurations for automating the Watchmaker RNA Library Prep Kit with Polaris Depletion: the Hamilton Microlab® NGS STAR MOA (Multiprobe Head On-Deck Thermalcycler Add On) and the Hamilton Microlab® NGS STAR LSA (Low Sample Assay) (Figure 2 and 3). Scripts are available to support the Watchmaker RNA Library Prep Kit with Polaris Depletion across both configurations, with minor differences to accommodate unique hardware features and capabilities of each (Table 1).

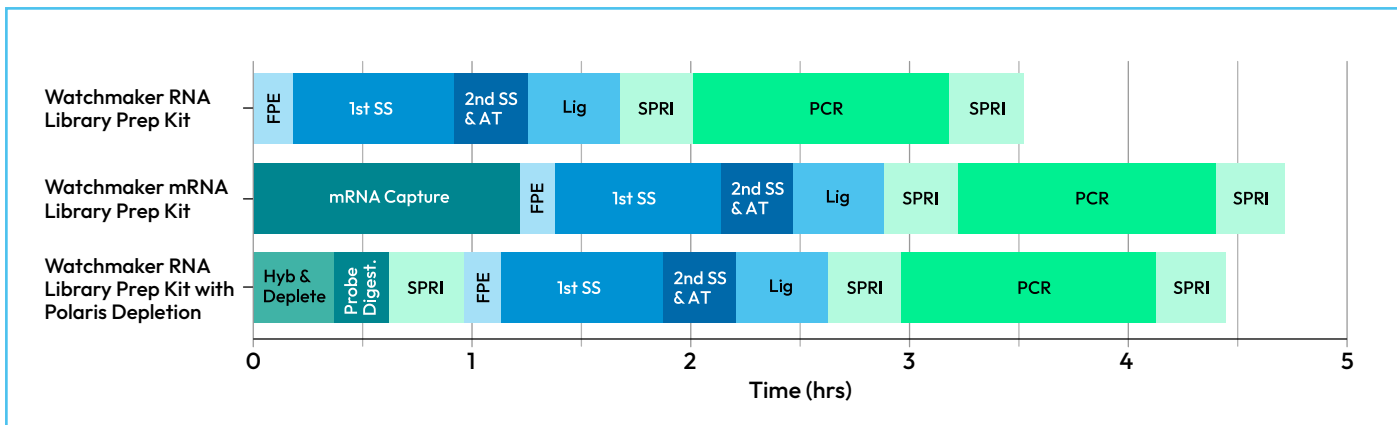
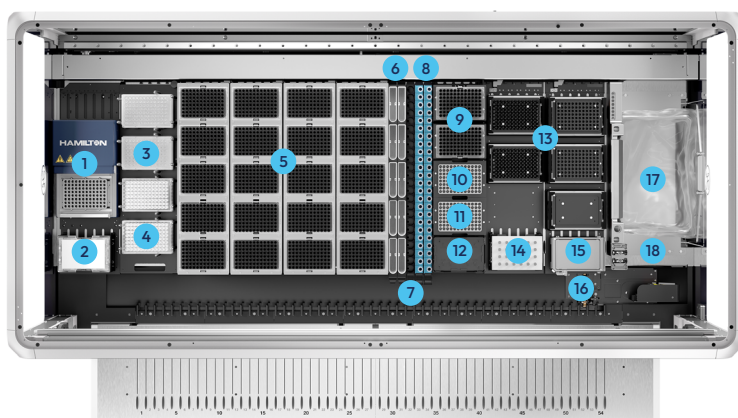


Figure 1: Truly single-day RNA library prep. The Watchmaker RNA portfolio offers three streamlined workflow options to address any RNA sequencing application and associated sample type. Reduced incubation times and fewer purification and handling steps makes all three workflows highly automatable and easily completed in a single workday.

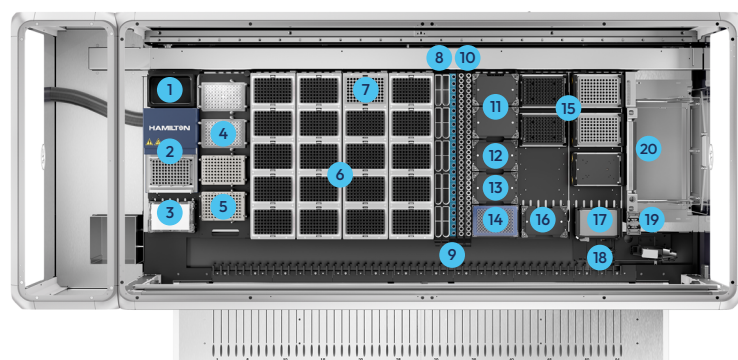
NGS STAR LSA Deck Layout



- 1 On-Deck Thermal Cycler (optional)
- 2 Lid Stack
- 3 Stacked 96-Well MIDI Plates
- 4 Stacked 96-Well PCR Plates
- 5 Conductive Tips
- 6 60 mL Reagent Troughs
- 7 15-17 mm Tubes
- 8 Microtubes
- 9 1 mL Conductive Tips
- 10 96-Well PCR Plate
- 11 96-Well PCR Plate
- 12 96-Well MIDI Plate
- 13 Heater Shakers
- 14 Ambion Post Magnet
- 15 Cold Plate Air Cooled (CPAC)
- 16 Autoload
- 17 Solid/Liquid Waste
- 18 CORE Gripper

Figure 2: Top View of Hamilton NGS STAR LSA Deck Layout.

NGS STAR MOA Deck Layout



- 1 Gravity Waste
- 2 On-Deck Thermal Cycler
- 3 Lid Stack
- 4 Stacked 96-Well MIDI Plates
- 5 Stacked 96-Well PCR Plates
- 6 Conductive Tips
- 7 Shifted CORE II Tip Adapter
- 8 60 mL Reagent Troughs
- 9 15-17 mm Tubes
- 10 Microtubes
- 11 96-Well PCR Plate
- 12 96-Well MIDI Plate
- 13 Deep-Well Plate
- 14 Alpaqua Magnum FLX without springs
- 15 Heater Shakers
- 16 DWP Module
- 17 Cold Plate Air Cooled (CPAC)
- 18 Autoload
- 19 CORE Gripper
- 20 Solid/Liquid Waste

Figure 3: Top View of Hamilton NGS STAR MOA Deck Layout.

Configuration	NGS STAR MOA	NGS STAR LSA
96 Multiprobe Head (MPH)	Yes	No
8 1000 µL Channels	Yes	Yes
On-Deck Thermal Cycler	Yes	Optional
Addition of Master Mix via Stamping	Yes	No
Parallel Processing of SPRI Cleanups	Yes	No
Process 1-96 Samples	Yes	Yes
Total Time to Process 96 Samples	< 7 Hours	< 10 Hours

Table 1: Hamilton Microlab NGS STAR MOA and LSA configuration comparison.

Both the MOA and the LSA scripts support dynamic runtime options, accommodating multiple workflows (Figure 4). Either configuration can process 1-96 samples in a single run, generating up to 96 high-quality, sequencing-ready libraries in less than 7 hours (MOA) or less than 10 hours (LSA).

Experiment Design & Parameters:

1. Low-Throughput (LT) Testing:

The initial performance of both automated methods was assessed by comparing a low-throughput sample run of 8 replicates of high-quality RNA control (Agilent Human Reference RNA, PN: 74000) at 50 ng inputs to manual library preparation (Table 2). The final library yields and sizes for automated runs were directly compared to the manually prepared libraries, looking for consistent and equivalent results.

2. High-Throughput (HT) Testing:

After low-throughput performance achieved acceptable results, 48 replicates of positive, high-quality RNA controls (Agilent Human Reference RNA, PN: 74000) were diluted to a total concentration of 50 ng per replicate alongside 48 negative, no template controls (NTCs) in a checkerboard pattern on both automated methods (Table 2). Final library QC results were analyzed for the absence of cross-contamination and common plate effects, alongside comparability to low-throughput and manual library preparations.

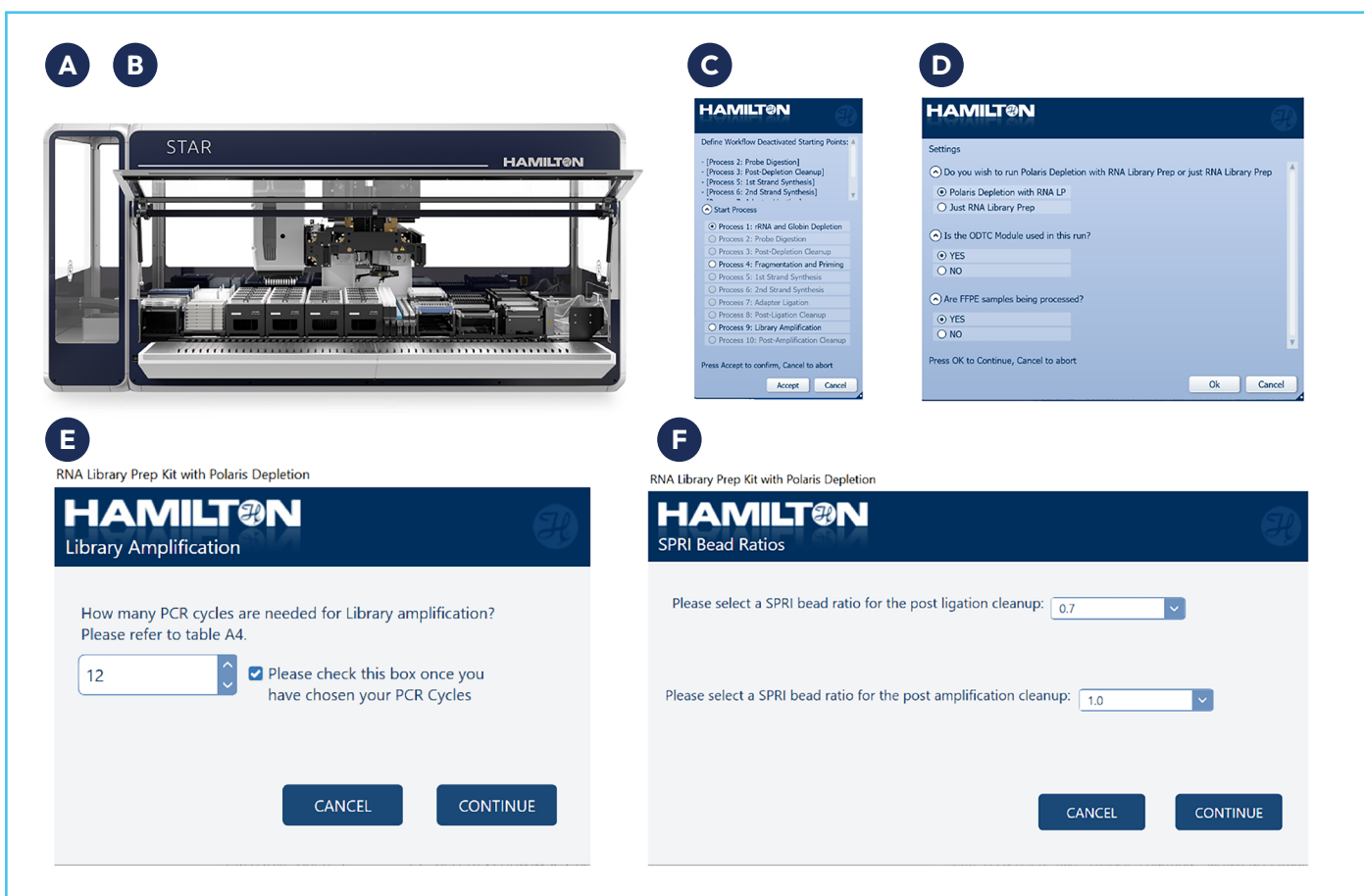


Figure 4: Dynamic User Options. The A) Hamilton Microlab® NGS STAR MOA enables flexibility and speed utilizing both its MPH and 8-independent channels. The B) Hamilton Microlab® NGS STAR LSA enables flexibility by utilizing its 8-independent channels to perform library preparation on 1-96 samples. The Watchmaker RNA Library Prep with Polaris Depletion script includes dynamic C) runtime user options for safe start/stop points, D) run conditions, E) PCR cycles, F) SPRI bead cleanup ratios, and more – supporting multiple workflows within a single automated method.

Configuration	Throughput	Sample Type	Sample Input (ng)	Fragmentation	Adapter Type	Adapter Concentration	PCR Cycles	Post-Ligation SPRI Ratio	Post-Amplification SPRI Ratio
NGS STAR MOA	LT	Agilent Human Reference RNA	50	85°C for 5 Minutes	xGen™ Stubby Adapter - UDI Primers	2 µM	11	0.7X	1.0X
	HT		1			0.2 µM	18		
	Ultra Low Input HT		50			Elevate Index and Adapter Kit	2 µM		
NGS STAR LSA	LT	Agilent Human Reference RNA	50	85°C for 5 Minutes	IDT Full Length Adapters	1 µM			
	HT								

Table 2: Experiment parameters for low-throughput and high-throughput testing.

3. Clinically Relevant High-Throughput Testing:

To further test the MOA configuration, a full plate of ultra-low input (1 ng), high-quality RNA samples and five NTCs were run to assess the method's performance at the lower input limit (Table 2). In parallel, manual and automated library preparations were performed, followed by sequencing of a subset of final libraries on an Illumina NextSeq 2000 using a P3 Flow Cell and 2 x 150 bp paired-end reads. Experiment conditions were chosen based on Watchmaker's User Guide.

Results & Discussion

To assess the performance of both automated methods, low-throughput runs were completed including 8 replicates of high-quality RNA controls each comparing results directly to manual processing when using identical experimental conditions. The results showed that both automated methods produced libraries with comparable yield and size to manual preparation, indicating expected performance of the automated methods and fitness for higher throughput testing (Table 3).

Next, high-throughput runs were completed for both automated methods with 48 replicates of high-quality RNA controls and 48 NTCs. All automated library prep results were highly reproducible with a coefficient of variation (CV) in concentration (ng/µL) of ≤ 10% and in size (bp) of ≤ 1% (Figure 5), while remaining comparable to manual library prep. Importantly, both methods were free of detectable cross-contamination, with no observable library yield in NTC wells (data not shown) and no notable plate effects. Final library yield and size CVs of < 10% indicate that the automated methods across both configurations offer the precision needed for high-throughput processing of WTS workflows. These results suggest that automation not only offers high reproducibility but also minimizes the risk of human error, ensuring that researchers can achieve reliable and accurate results consistently across experiments.

Configuration	Throughput	Automated Library Concentration (ng/µL)	Manual Library Concentration (ng/µL)	Automated Library Size (bp)	Manual Library Size (bp)
NGS STAR LSA	LT	35.8	41.7	400	386
NGS STAR MOA	LT	26.8	29.7	376	383

Table 3: Library QC metrics for low-throughput testing.

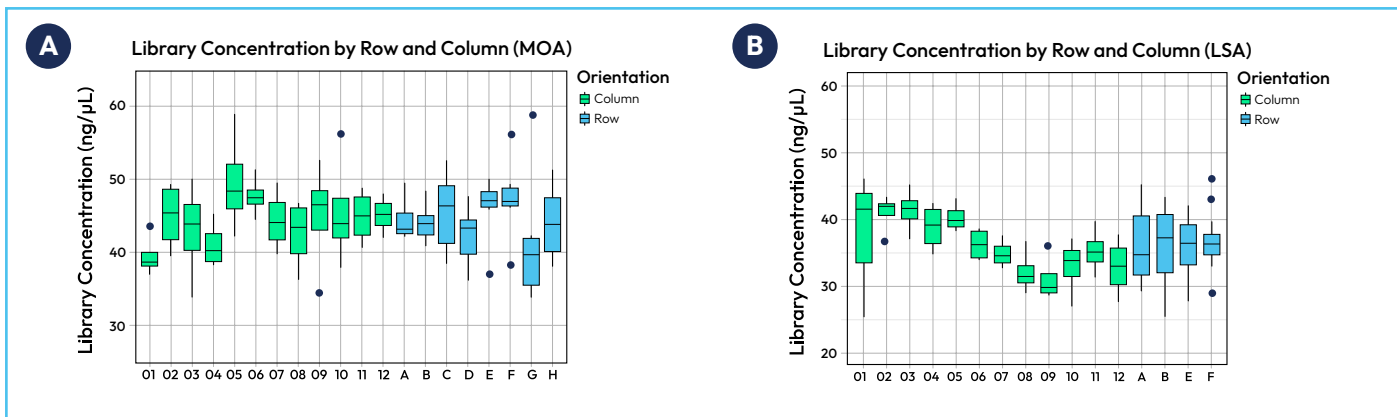


Figure 5: Final Library QC for High-Throughput Testing. A) Final library concentration for the MOA script across and down the plate. B) Final library concentration for the LSA script across and down the plate. Both plates were measured on the Qubit 4 Fluorometer using the 1X dsDNA High Sensitivity Assay.

Lastly, the third experiment assessed the NGS STAR MOA automated method with a clinically relevant sample input of 1 ng high-quality RNA. Final library QC and sequencing metrics were compared to manually prepared libraries to ensure the automated method's capabilities for producing high quality sequencing data even at ultra-low inputs. Both manual and automated approaches yield comparable results for key sequencing metrics such as base burndown (Figure 6), rRNA contamination (Figure 7A), and unique gene detection (Figure 7B), 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 even when automating workflows for ultra-low input samples.

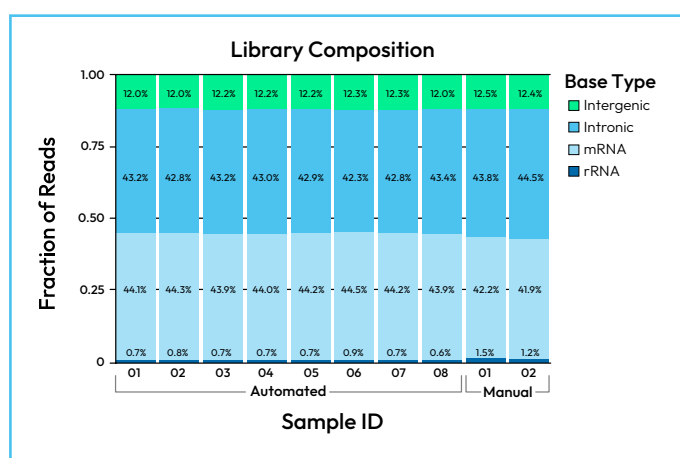


Figure 6: Base Burndown Comparison Between Preps. Comparison of base type distribution in RNA-seq libraries between automated and manual library preparation showing comparable base distribution of intergenic, intronic, mRNA, and rRNA bases. All sequencing data was downsampled to 20 million reads.

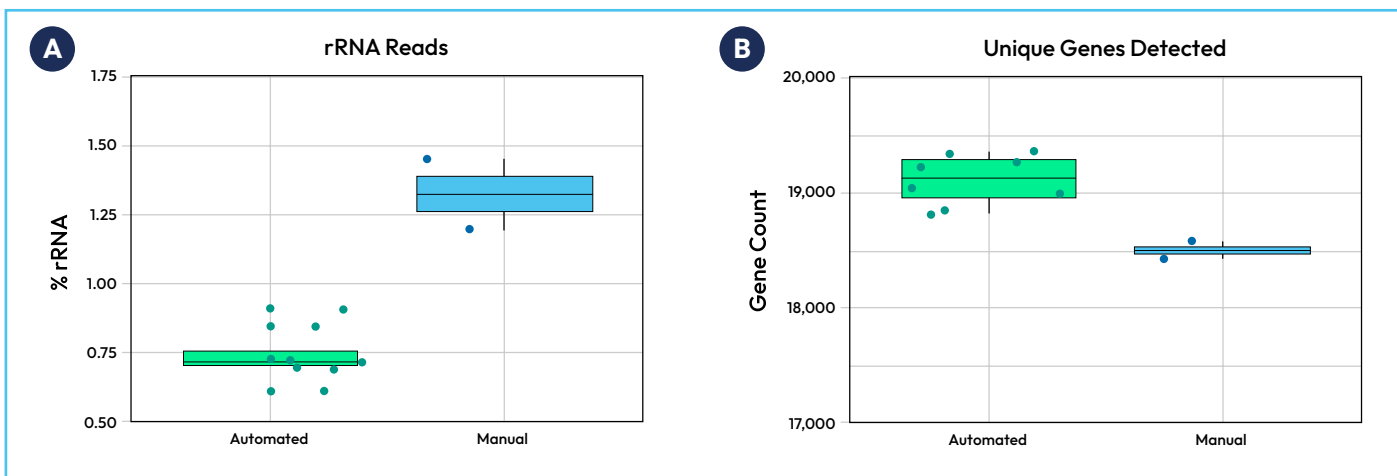


Figure 7: Assessment of rRNA Contamination and Unique Gene Detection. No significant differences were observed for A) residual rRNA and B) unique gene count between automated and manual library preparation methods.

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 and library prep chemistry (Figure 8). This 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.

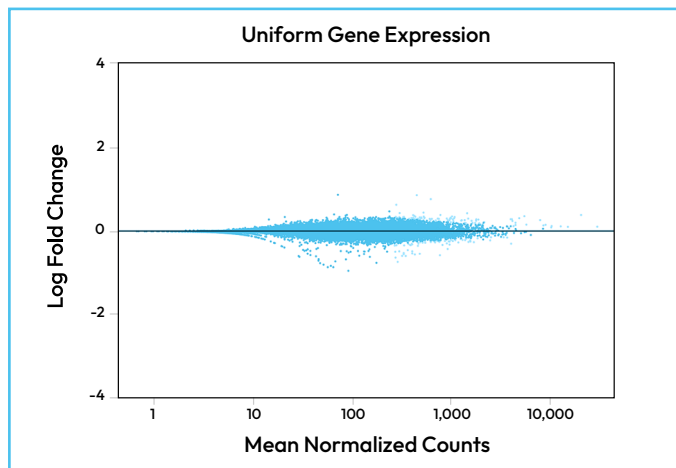


Figure 8: Differential Expression of Genes. Consistent gene expression profiles across low- and highly-expressed transcripts when comparing automated and manual library preparation methods.

Conclusion

In conclusion, the results from all three sets of experiments demonstrate that both LSA and MOA automation methods can successfully replicate the performance of manual RNA library preparation. The automated protocols are not only capable of meeting the stringent demands of RNA-Seq workflows but also provide substantial advantages in terms of efficiency, scalability, and minimizing human error.

While these findings are promising, future studies could further explore the performance of these automated methods with a broader, more challenging, range of sample types such as degraded RNA. Additionally, the integration of these automated systems into high-throughput workflows could offer opportunities to streamline large-scale RNA-Seq studies, opening new avenues for clinical research and diagnostics.

For further information on either automated method or any other technical inquiries please contact us via:

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