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APPLICATION NOTE No. 473

Automated Library Preparation Using the SMARTer® Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian on the epMotion® 5075t

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Abstract

Preparing robust, high-quality RNA sequencing (RNA-Seq) libraries is critical to the reliability and accuracy of the sequencing data. Automated library preparation minimizes sample loss and reagent usage and can also help eliminate a source of variability, providing efficiency, scalability, and consistency in sample preparation and resulting data quality. Eppendorf has partnered with Takara, to develop an automated workflow for the SMARTer® Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian on the Eppendorf epMotion 5075t automated liquid handling system.

The Pico v3 kit has been successfully used for transcriptome profiling from challenging sample types for a broad range of applications. The Pico v3 kit has been successfully used for high-quality stranded, total RNA-seq libraries with a high degree of reproducibility for challenging sample types and a broad range of applications. The data generated from this automated protocol show that this kit can be used with the epMotion 5075t to streamline such library preprations.

Introduction

Whole transcriptome RNA profiling using next-generation sequencing (NGS) is a powerful tool for biomarker discovery and is widely used in oncology, infectious disease, neurobiology, and ecology research.

Non-invasive liquid biopsies, circulating cell-free RNA (cfRNA) from plasma or other biological fluids, and archival formalin-fixed paraffin-embedded (FFPE) tissue specimens are the most common sample types used in oncology and clinical research applications. However, these samples are generally of poor quality and limited quantity, making high-quality NGS library preparation challenging.

SMARTer Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian (Pico v3) is ideally suited for efficient library preparation from such degraded, low input, and challenging samples. The kit uses a random priming and template-switching approach to generate cDNA with uniform coverage from picogram inputs (250 pg – 10 ng) of high-quality or degraded total RNA. The workflow also incorporates unique molecular identifiers (UMI) during reverse transcription to mitigate potential PCR errors and amplification biases, enabling quantitative differential expression analysis and accurate variant calling, especially for rare mutations. Efficient removal of ribosomal RNA and mitochondrial RNA reads is achieved using a proprietary ZapR™ depletion technology. Further, the directionality of the template-switching reaction preserves the strand orientation of the original RNA, making it possible to obtain strand-specific sequencing data for identifying regulatory anti-sense transcripts, providing more accurate and comprehensive transcriptome analysis.

The Pico v3 kit has been successfully used for transcriptome profiling from challenging sample types for a broad range of applications including FFPE[1], laser capture microdissection (LCM)[2], and cfRNA[3]. The kit has also been used for profiling long non-coding RNAs (lncRNAs) in human biofluids and extracellular vesicles (EVs)[4], and sequencing viriomes of RNA viruses like SARS-CoV-2 from wastewater samples[5].

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Preparing robust, high-quality RNA sequencing (RNA-Seq) libraries is critical to the reliability and accuracy of the sequencing data. Automated library preparation minimizes sample loss, reagent usage, and hand-on time, improving efficiency, scalability, and ease while preserving consistency in sample preparation and data quality. Eppendorf has partnered with Takara, to develop an automated workflow for the SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian on the Eppendorf epMotion 5075t automated liquid handling system. The data generated from the automated protocol show that high-quality stranded, total RNA-seq libraries can be prepared from 1 ng of input RNA with high reproducibility.

The workflow was readily automated using the epMotion 5075t and can process up to 48 samples at a time. The epMotion software has a user-friendly interface to guide the operator through the workflow, including placement of the labware, tools, and reagents. Before the run starts, an optical sensor verifies that all labware is correctly placed. The epMotion can be equipped with accessories for plate mixing, on-deck incubations, and magnetic bead separation. The automated platform allows for maximum walk away time.

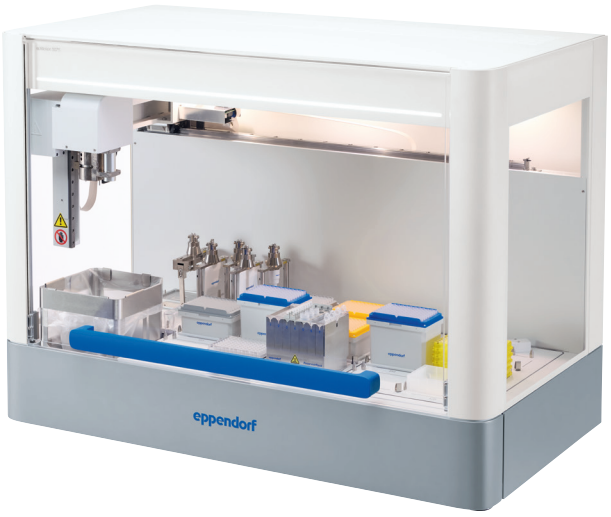


Figure 1:
Eppendorf epMotion 5075t automated liquid handling system

Material and Methods

epMotion Setup
Automated liquid handling was performed using the epMotion 5075t equipped with a thermal module, gripper tool, single-channel, and multichannel dispensing tools (50 µL and 300 µL). Magnetic bead separation was performed using Magnum FLX Magnet Adapter. The methods were divided into three sections (Figure 2). The method starts with 8 µl of total RNA. Most incubations were performed on-deck, except for the PCR steps. In addition, R probes were also denatured in a plate off deck in a Eppendorf thermocycler (Mastercycler® X50a). The epMotion will notify the user for when to prepare the probes and for when to add master mixes to the deck. On deck incubations were performed using a mineral oil overlay.

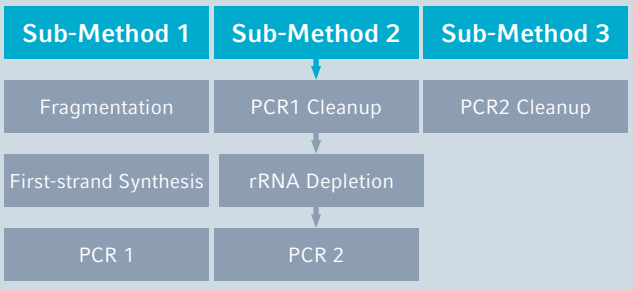
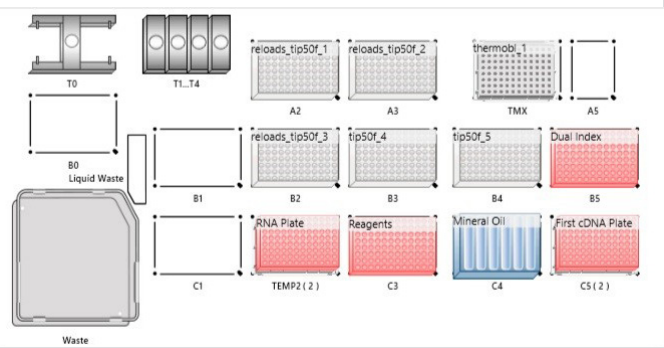


Figure 2: epMotion deck layouts for the three sub-methods

Material and Methods

RNA-seq library preparation
The Pico v3 kit is designed to work with picogram quantities (250 pg - 10 ng) of high-quality, partially degraded, or low-quality mammalian total RNA and includes indexed primers to prepare libraries for sequencing on the Illumina® platform. RNA-seq libraries were generated with 1 ng of human brain RNA (control provided in the kit) following the user guide recommendations on the epMotion 5075t platform. RNA (1 ng) was diluted into a final volume of 8 µL.
Quality and yield of all completed libraries were quantified using the Thermo Fisher Scientific Qubit® 2.0 Fluorometer and Agilent® 2100 Bioanalyzer®. Six of the sixteen libraries were subjected to Illumina sequencing. The libraries were pooled at equimolar ratios and sequenced on the MiSeq using the MiSeq v3 (2 x 76 bp) 150 cycle kit. Samples were sequenced to a depth of at least 3 M reads. The sequence matrices were then generated using Takara Bio Cogent™ NGS Analysis Pipeline v.2.0.

A



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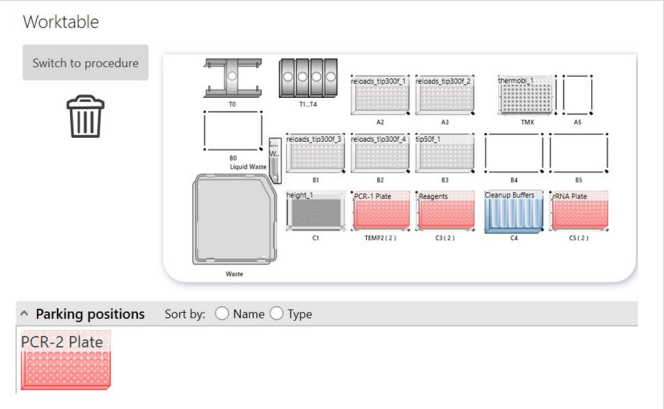


C

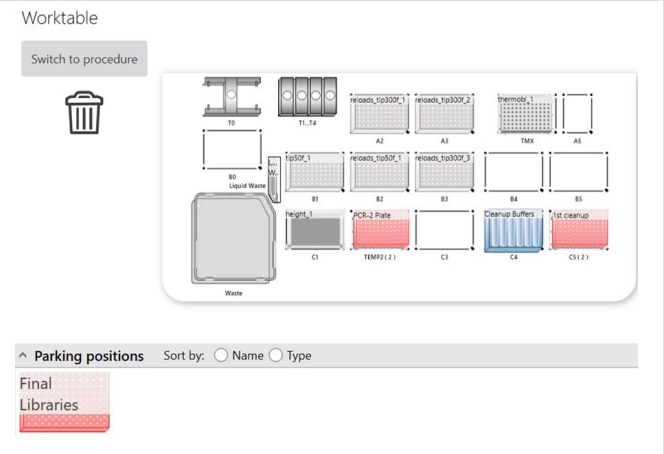


Figure 3: deck layouts for A) sub-method 1, B) sub-method 2, and C) sub-method 3

Results and Discussion

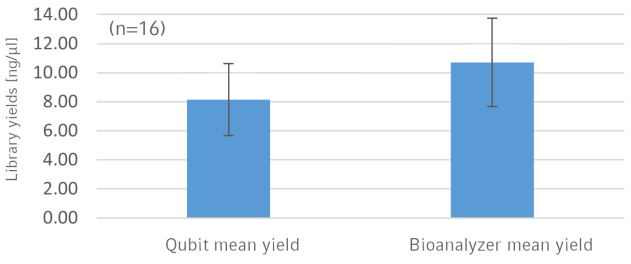
Robust automated library preparation with high yields and consistent size distribution – An important consideration for challenging low-input and clinically relevant samples

The robustness of the automated library preparation workflow was demonstrated by preparing 16 replicate libraries using 1 ng of human brain RNA. The initial assessment of the final libraries shows successful library prep with uniform yields (Figure 4A) and expected size distribution and mean peak at approximately 499 bp. The absence of adaptor and primer dimers indicate efficient library preparation and clean-up (Figure 4B).

Increase detection of biologically relevant transcripts with higher accuracy and strand of origin information

The high-quality Pico v3 sequencing libraries were prepared using the automated workflow. The libraries show precision in sequencing metrics with high on-target mapping, strand-specificity, expected UMI variability, and efficient rRNA depletion.

A



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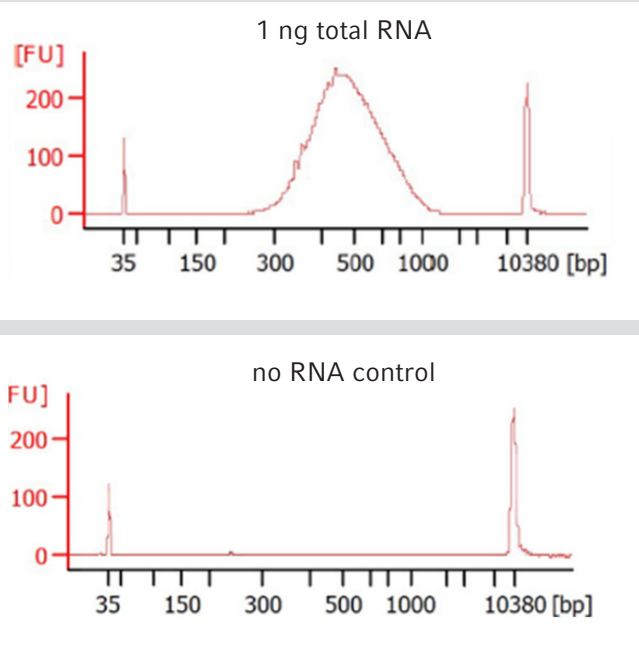

Figure 4: Assessment of library yield and quality, A) average yields for libraries generated with Pico v3 on the epMotion® 5075t automation platform, B) representative library traces for automated library preparation.

MiSeq v3 150cycle (22-25 million reads) 2 x 76, 12 pM input

Mapping statistics	QC criteria	A01	D01	G01	B02	E02	H02
Total reads	> 3,000,000	4584930	5163935	4891455	4681659	4542606	5157422
% Q30	≥ 85%	93.90%					
# Mapped reads	–	4373060	4958327	4655198	4373892	4341448	4925282
% Mapped reads	≥ 80%	95.4%	96.0%	95.2%	93.4%	95.6%	95.5%
# Mitochondrial reads	–	161015	197352	177570	163332	158896	180380
% Mitochondrial reads	≥ 7%	3.5%	3.8%	3.6%	3.5%	3.5%	3.5%
# Intergenic reads	–	231244	241817	246905	226430	215220	251860
% Intergenic reads	≥ 10%	5.04%	4.68%	5.05%	4.84%	4.74%	4.88%
#UMI	≥ 62,000	65,510	65,512	65,527	65,525	65,516	65,516
Strand specificity	≥ 90%	97.9%	98.2%	97.9%	97.9%	98.1%	98.0%
% rRNA reads (ribopicker)	≥ 20%	2.93%	5.13%	2.68%	4.14%	6.24%	3.77%

Table 1: Summary of the sequencing metrics for automated library preparation. Six of the sixteen libraries were sequenced on the Illumina MiSeq®. All the libraries sequenced pass the required QC matrix.

Conclusion

High-quality and consistent library preparation is a critical requirement for NGS success, reliability, and data accuracy. It is especially important for enabling biological discovery from clinically relevant samples which may be the low-input and variable quality. The Pico v3 kit is a powerful tool for RNA-Seq analysis, uncovering biomarkers including isoforms, gene fusions, and single nucleotide variants in both coding and non-coding RNA. The increased demand for high-quality RNA-Seq libraries in high-throughput laboratories has necessitated the development of robust, automated methods for library preparation. Takara has collaborated with Eppendorf to automate the SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian Kit on the Eppendorf epMotion 5075t. The experimental data show that the automated method performs within the kit specifications at the input amount tested. 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.

References

[1] Hilmi, M. et al., Whole-Transcriptome Profiling on Small FFPE Samples: Which Sequencing Kit Should Be Used?, Curr. Issues Mol. Biol. 2022; 44(5), 2186-2193
[2] Almeda, D. and Turecki, G., Profiling cell-type specific gene expression in post-mortem human brain samples through laser capture microdissection, Methods. 2022; 207:3-10
[3] Chang, A. et al., Circulating Cell-Free RNA in Blood as a Host Response Biomarker for the Detection of Tuberculosis, medRxiv (2023): 2023-01
[4] Everaert, C. et al., Performance assessment of total RNA sequencing of human biofluids and extracellular vesicles, Sci Rep. 2019; 9(1):17574
[5] Wyler, E. et al., Comprehensive profiling of wastewater viromes by genomic sequencing, bioRxiv (2022): 2022-12

Takara Bio Ordering Information

Description	Catalog #
SMARTer® Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian	634485, 634486, 634487, 634488
Unique Dual Index Kits (1-96), (97-192), (193-288), (289-384), (1-24)	634752, 634753, 634754, 634755, 634756

Eppendorf Ordering Information

Product	Order no. (INT)	Order no. (ENA)
epMotion® 5075t	5075000042	5075000042
Thermal module on position C2	5075757508	5075757508
TS 50 Dispensing Tool	5280000010	960001010
TM 50-8 Dispensing Tool	5280000215	960001044
TS 300 Dispensing Tool	5280000037	960001028
TM 300-8 Dispensing Tool	5280000231	960001052
Gripper	5282000018	960002270
Thermoblock PCR 96 OC	5075751666	5075751666
Thermoadapter PCR 96 (3)	5075787008	960002199
Reservoir rack 7	5075754002	960002148
epT.I.P.S.® Motion, 50 µL, filtered, PCR clean	0030014413	0030014413
epT.I.P.S.® Motion, 300 µL, filtered, PCR clean	0030014456	0030014456
epMotion® Reservoirs, 10 mL	0030126521	0030126521
epMotion® Reservoirs, 30 mL	0030126505	960051009
epMotion® Reservoir, 400 mL	5075751364	5075751364
Eppendorf twin.tec® PCR Plate 96 LoBind®, semi-skirted	0030129504	0030129504
Eppendorf twin.tec® PCR Plate 96 LoBind®, skirted	0030129512	0030129512
Eppendorf MAGNUM FLX® Magnet	5075751836	960066124
Waste bags	5075752034	5075752034

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