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Life Sciences

Automation of Illumina RNA Prep with Enrichment kit on the Biomek NGeniuS Next Generation Library Prep System

Abstract

As genome sequencing and data analysis methods become more accessible, more laboratories are exploring NGS (Next Generation Sequencing) as a research tool. RNA sequencing applications (RNA-Seq) have allowed researchers to investigate allele-specific expression, fusion detection, biomarker screening, and pathogen identification. In this paper, we detail an automated process for the Illumina RNA Prep with Enrichment library preparation kit. The workflow is divided into two separate App Templates. The first, Illumina RNA Prep with Enrichment: Library Prep allows the users to prepare between 4 and 24 samples into sequence-ready RNA libraries on the Biomek NGeniuS Library Prep System. The second App Template, Illumina RNA Prep with Enrichment: Target Enrichment, allows the user to enrich between 4 and 16 library pools as either 1-plex or 3-plex pools on the Biomek NGeniuS Library Prep System. Both App Templates require minimal interaction from the user.

Introduction

The Illumina RNA Prep with Enrichment assay is a comprehensive NGS research-use-only assay that allows for the construction and enrichment of RNA-Seq libraries from either high-quality RNA or RNA isolated from formalin-fixed, paraffin-embedded (FFPE) samples. A variety of different enrichment panels including the Illumina Exome Panel and the Illumina RNA Pan-Cancer panels are available for applying the assay to whole exome or cancer genomics research. Additionally, panels are available for using the assay to characterize a variety of respiratory viral pathogens, such as Covid-19 and RSV.

The **Illumina RNA Prep with Enrichment App** Template on the Biomek NGeniuS Next Generation Library Prep System enables the generation of libraries compatible with Illumina sequencing platforms. The App Template is split into two parts, the Library Preparation App and the Target Enrichment App.

The Illumina RNA Prep with Enrichment: Library Preparation App starts with the 'Normalize Samples' section then runs cDNA synthesis to clean up of tagmented libraries. A range between 4 to 24 libraries are produced in a single batch run. The App is optimized for use of 10–100 ng of purified total RNA sample input. Lower input amounts or use of RNA input from degraded or FFPE samples can reduce library yield. The user is required to specify the concentration of the starting material to enable normalization. The number of Tagmentation PCR cycles can also be adjusted between 14 and 17 cycles, and bead drying time following ethanol washes can be set between 2 and 5 minutes. 80% ethanol wash volumes have been reduced from 175 µL to 50 µL.

Library quantification, normalization and enrichment pooling steps are to be performed off-deck.

The **Illumina RNA Prep with Enrichment: Target Enrichment App** runs from capture probe hybridization to clean up of enriched libraries and allows the user to produce between 4 to 16 pools in a single batch run. The user may utilize 200 ng one-plex enrichment libraries or 600 ng three-plex enrichment libraries as sample input. The hybridization time has been limited to the minimum time listed in the manual protocol (90 minutes) to reduce application run time and cannot be changed. 80% ethanol wash volumes have been reduced from 175 µL to 50 µL. The post enrichment amplification PCR program extension time has been increased from 30 seconds to 60 seconds to improve assay performance.

The App templates were designed using the Illumina RNA Prep with Enrichment (L) Tagmentation Reference guide (Document # 1000000124435 v04). The App Templates utilize the Illumina RNA Prep with Enrichment (L) Tagmentation (96 Samples) kit (Illumina Part Number 20040537) in conjunction with the IDT® for Illumina® DNA/RNA UD Indexes Sets A, B, C, or D (Illumina Part Numbers 20027213, 20027214, 20042666, or 20042667) or the Illumina DNA/RNA UD Indexes Sets A, B, C or D (Illumina Part Numbers 20091654, 20091656, 20091658, 20091660). Figure 1 displays steps of the chemistry workflow covered by each of the apps.

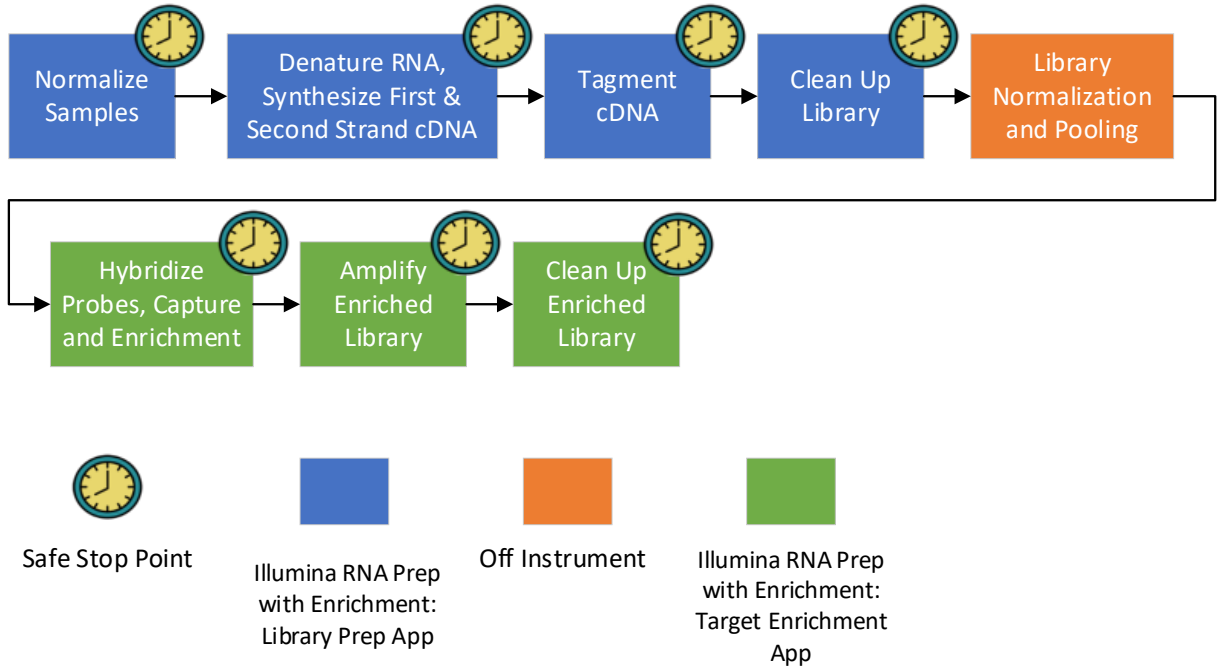

Figure 1: Workflow of the Illumina RNA Prep with Enrichment Apps for Biomek NGeniuS system.

In this application note, demonstration of the Illumina RNA Prep with Enrichment: Library Preparation App and Illumina RNA Prep with Enrichment: Target Enrichment App is shown for 10 ng and 100 ng inputs utilizing the Illumina Whole Exome (CEX) panel or the Illumina RNA Pan-Cancer (RPO) panel.

1. Materials and Methods

1.1 Run Setup

Runs using the **Illumina RNA Prep with Enrichment: Library Preparation App** were set up in the Biomek NGeniuS system customer portal. This App has four settings for the user to interact with. The user may specify the Library Prep Input Mass to be used in library preparation with a valid range from 10 ng to 100 ng. Input RNA using this target mass will be transferred from the source reaction vessel (RV) to a new reaction vessel and normalized to the correct starting volume as part of the normalization process included in the Illumina RNA Prep with Enrichment: Library Preparation App. The user may select the Index Plate Set being used (Set A, B, C, or D), as well as the number of cycles being used for the post Tagmentation PCR (14 to 17 cycles) and the Bead Dry Time (2 to 5 minutes).

After completing the runs using the Illumina RNA Prep with Enrichment: Library Preparation App, the libraries would be quantified and pooled (if running 3-plex enrichments) manually.

Following off-deck quantification and pooling if required, runs using the **Illumina RNA Prep with Enrichment: Target Enrichment App** were set up in the Biomek NGeniuS System customer portal. This App has a single setting for the user to interact with, Bead Dry Time, which allows the user to vary the time for bead drying during cleanup from between 2 and 5 minutes.

A summary of experiment details is presented in Table 1. For the demonstration runs either Agilent Human Universal Reference RNA (Part Number 740000) or Thermo Human Universal Reference RNA (Part Number QS0639) was used.

Experiment	Library Number	Pool Number	Input Material	Input Mass (ng)	Panel	App Settings	Instrument	Sequencing	Analysis Workflow
1	16	6 (3-plex and 1-plex)	Agilent UHR	100	CEX	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Illumina UK	(CEX): NovaSeq 6000 S4 flowcell 2x100 bp PE run (RPO): NovaSeq 6000 SP flowcell 2x74 bp PE run	FASTQ Toolkit v2.2.0 (Down-sampled to 25 M reads) Enrichment v3.1.0
2	16	6 (3-plex and 1-plex)	Agilent UHR	10	CEX	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Illumina UK	NovaSeq 6000 SP flowcell 2x100 bp PE run	FASTQ Toolkit v2.2.0 (Down-sampled to 25 M reads) Enrichment v3.1.0
3	4	4 (1-plex)	Thermo UHR	100	CEX	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Illumina San Diego	NovaSeq 6000 S2 flowcell 2x100 bp PE run	FASTQ Toolkit v2.2.0 (Down-sampled to 25 M reads) Enrichment v3.1.0
4	24	8 (3-plex)*	Agilent UHR	100	RPO	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Illumina UK	NovaSeq 6000 S4 flowcell 2x74 bp PE run	FASTQ Toolkit v2.2.0 (Down-sampled to 4 M reads) RNA Seq Alignment v1.1.1
5	15	15 (1-plex)	Thermo UHR	10	RPO	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Illumina San Diego	NovaSeq 6000 S2 flowcell 2x74 bp PE run	FASTQ Toolkit v2.2.0 (Down-sampled to 4 M reads) RNA Seq Alignment v1.1.1
6	24	8 (3-plex)*	Agilent UHR	10	RPO	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Illumina UK	NovaSeq 6000 SP flowcell 2x74 bp PE run	FASTQ Toolkit v2.2.0 (Down-sampled to 4 M reads) RNA Seq Alignment v1.1.1
7	16	6 (3-plex and 1-plex)	Agilent UHR	100	CEX	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Beckman Coulter	NovaSeq 6000 S4 flowcell 2x100 bp PE run	FASTQ Toolkit v2.2.0 (Down-sampled to 25 M reads) Enrichment v3.1.0

Table 1. Summary of experiment conditions and App Settings.

1.2 Reagents, Consumables, and Equipment

Reagents	Manufacturer	Part Number
Illumina® RNA Prep with Enrichment, (L) Tagmentation (96 Samples)	Illumina	20040537
Illumina® DNA/RNA UD Indexes Set A-D, Tagmentation (96 Indexes, 96 Samples)	Illumina	20091654, 20091656, 20091658, 20091660
Illumina Exome Panel – Enrichment Oligos Only	Illumina	20020183
TruSight RNA Pan-Cancer Oligo Panel	Illumina	20046104
NovaSeq 6000 S4 Reagent Kit v1.5 (200 cycles)	Illumina	20028313
NovaSeq 6000 S2 Reagent Kit v1.5 (200 cycles)	Illumina	20028315
NovaSeq 6000 SP Reagent Kit v1.5 (200 cycles)	Illumina	20040719
Qubit 1X dsDNA HS Assay Kit	Thermo Fisher Scientific	Q33231
PCR grade Water	Invitrogen-Life Technology	10977-015
Ethanol	Thermo Fisher Scientific	BP2818-500

Table 2. Reagents used for Illumina RNA Prep with Enrichment library preparation and sequencing on Illumina sequencer.

Equipment	Manufacturer
Biomek NGeniuS Sample Prep System	Beckman Coulter Life Sciences
NovaSeq 6000 Sequencer	Illumina
Qubit	Thermo Fisher Scientific

Table 3. Equipment used in sample preparation and processing of Illumina RNA Prep with Enrichment libraries.

Consumable	Manufacturer/ Part Number
Foil Plate Seals	Beckman Coulter 538619
Biomek NGeniuS Reaction Vessel, 24 Well	Beckman Coulter C62705
Biomek NGeniuS Lid, 24 Well	Beckman Coulter C62706
Biomek NGeniuS Bulk Reservoirs, 25 mL/Section	Beckman Coulter C62707
Biomek NGeniuS Seal Pad	Beckman Coulter C70665
1025 µL Conductive Filtered Tips, Case	Beckman Coulter C59585
70 µL Conductive Filtered Tips, Case	Beckman Coulter C62712
Empty Tip box 1025 µL, Case	Beckman Coulter C70672
Empty Tip box 70 µL, Case	Beckman Coulter C70673

Table 4. Biomek NGeniuS System consumables quantity required for sample processing.

1.3 NGeniuS Produced Libraries and Sequencing

RNA samples (Table 1) were processed on the Biomek NGeniuS system using reagents, equipment, and consumables detailed in Tables 2, 3, and 4. After completing the runs using the Illumina RNA Prep with Enrichment: Library Preparation App, the libraries were characterized on the 4200 TapeStation using D1000 High Sensitivity ScreenTape (Agilent) and quantified using the Qubit 1X ds DNA High Sensitivity Assay Kit (Thermo) before being transferred as single-plex enrichments to a new reaction vessel or combined and concentrated to create 3-plex enrichments prior to transfer to the new reaction vessel. Libraries were then

processed using the Illumina RNA Prep with Enrichment: Target Enrichment App. Following enrichment, the libraries were again characterized on the 4200 TapeStation using D1000 High Sensitivity ScreenTape (Agilent) and quantified using the Qubit 1X ds DNA High Sensitivity Assay Kit (Thermo) prior to sequencing. Libraries were sequenced on an Illumina NovaSeq 6000 using either an SP, S2, or S4 flowcell and either a 2x100 bp paired end run for CEX panel libraries or a 2x74 bp paired end run for the RPO panel libraries. CEX panel libraries were down-sampled to 25 million reads using the FASTQ Toolkit App (v2.2.0) and then analyzed using the Enrichment App (v3.1.0) with 150 bp padding on Illumina BaseSpace. RPO panel libraries were down-sampled to 4 million reads using the FASTQ Toolkit App (v2.2.0) and then analyzed using the RNASeq Alignment App (v1.1.1) with 150 bp padding on Illumina BaseSpace. Data was subsequently visualized using JMP (version 14.2).

2. Results & Discussion

A total of seven Biomek NGeniuS system runs were performed during the course of demonstration. Following the completion of the runs with the Illumina RNA Prep with Enrichment: Library Preparation App pre-enrichment library concentrations were found to be significantly higher than the required yield of 26.6 ng/µL lower spec limit for CEX panel libraries (n = 51, mean = 203 ng/µL, standard deviation = 57.1 ng/µL) and for RPO panel libraries (n = 64, mean = 279 ng/µL, standard deviation = 96.1 ng/µL). The two negative controls included RPO panel library runs 4 and 6 failed to generate any detectable library according to Qubit. Pre-Enrichment concentrations for each panel are shown in Figure 2.

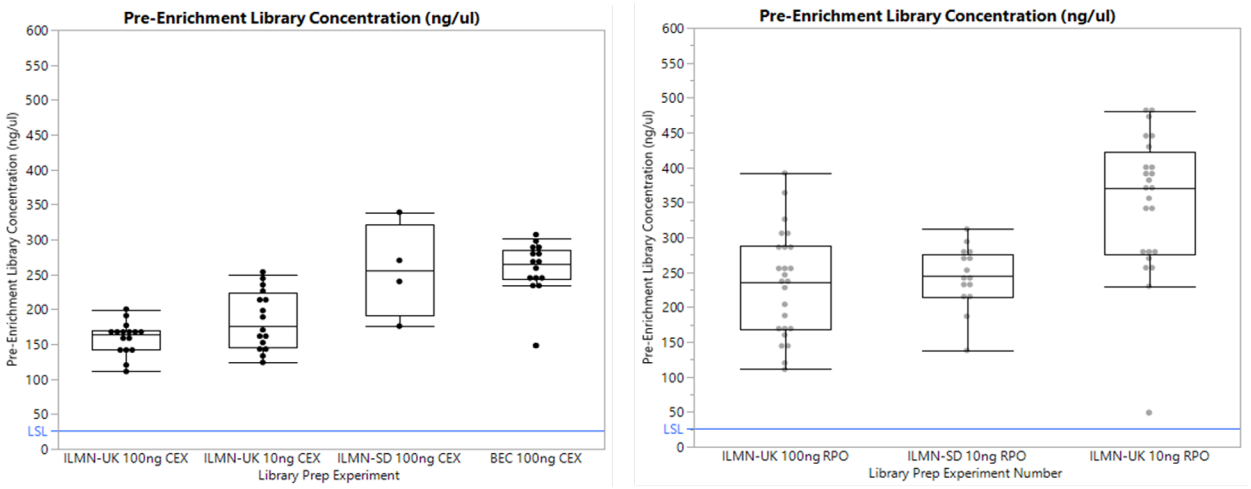

Figure 2: Pre-Enrichment library concentrations for CEX panel (left) and RPO panel (right) Illumina RNA Prep with Enrichment libraries prepared on the Biomek NGeniuS system.

Libraries were pooled as needed and run through the Illumina RNA Prep with Enrichment: Target Enrichment App as outlined in Table 1. Post-Enrichment library concentrations were significantly higher than what was required for sequencing for both the CEX panel libraries (n = 51, mean = 259 nM, standard deviation = 96.8 nM) and the RPO panel libraries (n = 64, mean = 197 nM, standard deviation = 59.6 nM). Example TapeStation traces for both Pre-Enrichment and Post-Enrichment CEX panel libraries are shown in Figure 3.

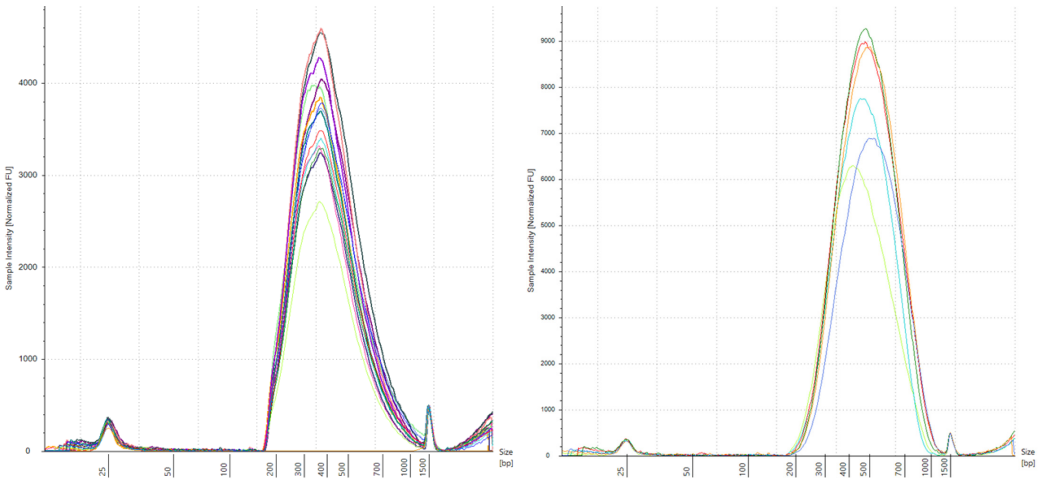

Figure 3: Pre-Enrichment Library Agilent TapeStation traces for 15 libraries and one negative control (left) and Post-Enrichment Agilent TapeStation traces for five 3-plex enrichment pools and one 1-plex enrichment pool (right) from the BEC 100 ng CEX Panel Run on the Biomek NGeniuS system. of the Illumina RNA Prep with Enrichment Apps for Biomek NGeniuS system.

Sequencing and analysis were performed for each Biomek NGeniuS system run as outlined in Table 1. Average percent aligned reads across all CEX panel libraries were higher than the 90% lower limit (n = 51, mean = 98.67%) as were the RPO panel libraries (n = 64, mean = 92.4%). Average per batch percent padded read enrichment of 90% or higher was set as a passing criteria by Illumina. Across all libraries, average percent padded read enrichment was higher than 90% for both the CEX panel libraries (n = 51, mean = 91%) and RPO panel libraries (n = 64, mean = 96.8%) despite the significant size differences in the panels (CEX = 45 Mb, RPO = 0.4 Mb) and the different analysis apps that were used for each panel. Average per batch percent padded read enrichment was 90% or higher for each batch run. Percent padded read enrichments for each panel are shown in Figure 4.

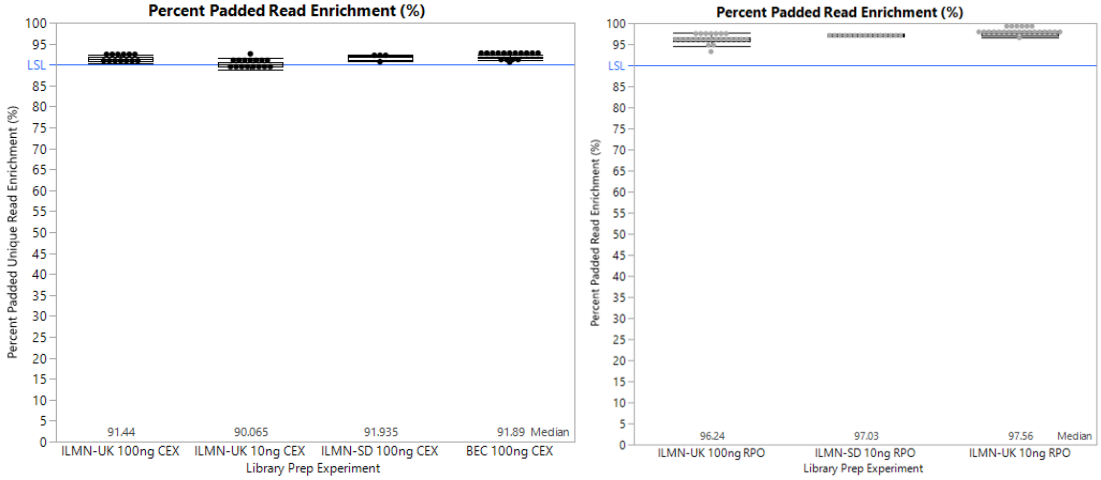

Figure 4: Percent padded read enrichment for CEX panel (left) and RPO panel (right) Illumina RNA Prep with Enrichment libraries prepared on the Biomek NGeniuS system.

Summary

We demonstrated the Illumina RNA Prep with Enrichment Apps on the Biomek NGeniuS Next Generation Library Prep System produce high-quality libraries from a variety of input mass and enrichment panels suitable for sequencing on Illumina sequencing platforms.

References

1. Illumina RNA Prep with Enrichment kit Guide (Document # 1000000124435 v04)

2024-GBL-EN-106524-V1