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Life Sciences

Automation of Illumina DNA PCR-Free Prep Kit with Plate-based Unique Dual Indices on the Biomek NGeniuS Next Generation Library Prep System

Abstract

Library preparation steps involved in Next Generation Sequencing (NGS) of DNA often include the use of Polymerase Chain Reaction (PCR) to amplify small quantities of DNA. This amplification step allows the generation of sufficient quantities of DNA necessary for sequencing. PCR amplification, however, can introduce bias by swamping out tumor-normal variants and result in uneven coverage during Whole Genome Sequencing (WGS). The Illumina DNA PCR-Free Prep, Tagmentation library preparation kit uses On-Bead Tagmentation and does away with the PCR amplification step to generate libraries suitable for sequencing. In this paper, we detail an automated process using the Biomek NGeniuS System for the Illumina DNA PCR-Free Prep, Tagmentation kit using plate-based Unique Dual Index primers. The Biomek NGeniuS Library Prep System and Illumina DNA PCR-Free Prep App will process between 4 and 24 samples from start to finish, with minimal interaction from the user, for DNA input masses between 100 ng and 2000 ng.

This application note describes the preparation of libraries from 100, 300, and 2000 ng input genomic DNA (gDNA) using the Illumina-developed DNA PCR-Free Prep App on the Biomek NGeniuS system. Sequencing results from library preparation show greater than 40x average autosomal coverage with greater than 97% autosome callability and greater than 0.87 mean normalized coverage at GC rich regions.

Introduction

The costs of WGS continue to fall, bringing this tool within reach of small and large research labs. Historically, library preparation for WGS frequently involved fragmenting genomic DNA (gDNA) to approximately 400 bp to 800 bp in length, ligating on adapters that allow the fragments to bind to the surface of the flowcell, and then using PCR to amplify the adapter-ligated fragments to generate enough sequence-ready libraries. However, this can result in uneven coverage across regions of high GC content. PCR-free library preparation shows more uniform coverage and fewer read errors in short tandem repeats as opposed to PCR-based workflows.[1] Illumina's DNA PCR-Free Prep, Tagmentation kit accommodates DNA input masses from 100 to 2000 ng kit in the Standard Input protocol.[2] The On-Bead Tagmentation chemistry fragments the DNA to appropriate size and adds adapter sequences, after which unique indices are appended for sample identification during pooled sequencing.

The DNA PCR-Free Prep App on the Biomek NGeniuS System was developed by Illumina following the Standard Input protocol for input mass ≥ 100 ng and is here demonstrated by Beckman Coulter Life Sciences at 100 ng, 300 ng, and 2000 ng. The 24-sample kit configuration aligns with the throughput of the Biomek NGeniuS Workstation, preparing up to 24 dual-indexed paired-end-stranded libraries from extracted gDNA. The DNA PCR-Free Prep App on the Biomek NGeniuS system begins with an optional sample mass normalization followed by automation of library preparation and cleanup, requiring no user interactions after the initial setup, and resulting in adapter-ligated fragments that are ready for quantification, pooling, and sequencing **(Figure 1)**.

Figure 1. Workflow for Illumina DNA PCR-Free Prep protocol on the Biomek NGeniuS system. The blue arrows indicate the steps that are done on the instrument; the red arrows indicate the steps that are not.

Materials and Methods:

Human genomic DNA samples (Coriell Institute for Medical Research, Homo sapiens – CEPH/Utah pedigree NA12878) were quantified using a Qubit 1X dsDNA BR Assay Kit on an Invitrogen Qubit 4 Fluorometer with the dsDNA assay type, and diluted to an initial starting concentration suitable for the Biomek NGeniuS system.

To reduce manual pipetting errors, the system enforces input nucleic acid concentration within 100X of the concentration required by the library preparation kit. This reduces the pipetting of small volumes of highly concentrated DNA, thereby reducing chances of manually introduced errors between samples.

When samples are ready, the run is set up in the Biomek NGeniuS customer portal. The Illumina DNA PCR-Free Prep App v1.0.0 has settings which allow the operator to select the library prep input mass between 100 and 2000 ng per the Standard Protocol, Index Set, and purification bead dry time. The App allows for using either the IDT® for Illumina® DNA/RNA UD Index sets or Illumina® DNA/RNA UD Index sets. A summary of experiment details and parameters is presented in Table 1. All experiments presented here used the IDT® for Illumina® DNA/RNA UD Indexes Set A, 96-well index plate.

Experiment	1	2	3
Sample type	H. sapiens gDNA	H. sapiens gDNA	H. sapiens gDNA
Sample count	9*	24**	4*
Library Prep Input Mass (ng)	100	300	2000
Bead Dry Time (min)	2	2	2
Pooling strategy	By mass	By volume	By mass
Sequencer	NovaSeq 6000	NovaSeq 6000DX***	NovaSeq 6000
Flow cell	S2	S4	S2

Table 1. Summary of experiment conditions

* Included 1 negative control. ** Included two negative controls. *** NovaSeq 6000DX was run in RUO mode.

Samples of gDNA were processed on the Biomek NGeniuS system using reagents, equipment, and consumables detailed in Tables 2, 3, and 4. Reagent HP3 (2N NaOH) was manually diluted and presented to the Biomek NGeniuS system in a 2.0 mL Sarstedt tube per the Work Aid instructions. Illumina Purification Beads (IPB) were presented to the Biomek NGeniuS system in a 5.0 mL Sarstedt tube per the Work Aid instructions. Normalization of input nucleic acid was automated on the instrument by diluting an aliquot of the sample to the input volume required by the library preparation kit to arrive at the correct starting concentration, and the App immediately proceeded into library construction.

Reagents	Manufacturer	Part Number
Illumina® DNA PCR-Free Prep, Tagmentation (24 Samples)	Illumina	20041794
IDT® for Illumina® DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples)	Illumina	20027213
Qubit 1X dsDNA BR Assay Kit	Thermo Fisher Scientific	Q33266
Qubit ssDNA Assay Kit	Thermo Fisher Scientific	Q10212
NovaSeq™ Reagents	Illumina	20028314
PCR grade Water	Invitrogen-Life Technologies	10977-015
Ethanol, 100%	General supplier	N/A

Table 2. Reagents used in preparation of libraries with Illumina DNA PCR-Free Prep kit and sequencing on Illumina NovaSeq 6000.

Equipment	Manufacturer
Biomek NGeniuS Sample Prep System	Beckman Coulter Life Sciences
NovaSeq 6000 or 6000DX Sequencer	Illumina
Qubit 4 Fluorometer	Thermo Fisher Scientific

Table 3. Equipment used in sample preparation and processing of Illumina DNA PCR-Free Prep.

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
2.0 mL Conical Tube	Sarstedt 72.664
5.0 mL Conical Tube	Sarstedt 60.611

Table 4. Consumables required for sample processing.

All Biomek NGeniuS system's prepared libraries were quantified using a Qubit ssDNA Assay Kit on an Invitrogen Qubit 4 Fluorometer with the Oligo ssDNA assay type.

The resulting 8 libraries from the 100 ng input run, excluding the negative control, were diluted to 2 nM using Resuspension Buffer (RSB) and pooled. The resulting 3 libraries from the 2000 ng input run, excluding the negative control, were diluted to 2 nM using RSB and pooled. The resulting 22 libraries from the 300 ng input run, excluding the negative controls, were pooled by volume using 9 µL of each library. The concentration of the resulting pool was measured using a Qubit ssDNA Assay kit. A 2 nM dilution of the pool was prepared using RSB as diluent. The three, 2 nM normalized library pools were sent to Illumina Solutions Center, Baltimore, MD, USA for sequencing.

The 100 ng and 300 ng sample pools were individually denatured and diluted to 400 pM for an S2 flow cell following the NovaSeq 6000 Protocol A for standard loading. The 2000 ng sample pool was denatured and diluted to 400 pM for an S4 flow cell following the NovaSeq 6000 Protocol A for standard loading. A 1% PhiX spike-in was included for all three sequencing runs. Sequencing used a run configuration of 151|10|10|151 read cycles. Data were analyzed via the DRAGEN™ Germline app in the BaseSpace™ Sequence Hub, with alignment against the Human UCSC hg38 Alt-Masked v3 reference genome assembly.

Results and Discussion:

Only three setup interactions with the Biomek NGeniuS system were necessary to generate libraries with the Illumina DNA PCR-Free Prep App. First, consumables, bulk reagents, and the 96-well plate containing indices were placed on the instrument. The Biomek NGeniuS system verified the presence of consumables, checked the liquid levels on the bulk reagents, and aliquoted indices to the cold storage location. Second, ambient storage locations were populated, and the remaining reagents were presented to the system via the Carousel. These reagents were aliquoted to storage locations by the Biomek NGeniuS system. Finally, the gDNA samples were placed on the system, and library preparation was initiated. The prepared libraries were presented to the operator at the end.

At all tested input concentrations, libraries of sufficient concentration (i.e., ≥ 2 nM as measured by Qubit ssDNA) for the NovaSeq 6000 standard workflow were produced, and higher input concentrations resulted in greater library yields. The negative controls resulted in no measurable yield.

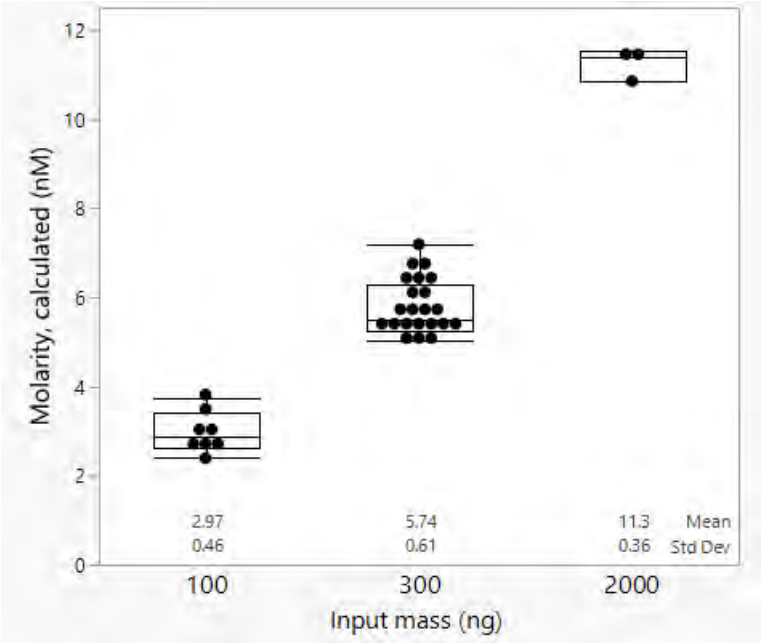


Figure 2. Yield from processing H. sapiens gDNA on the Biomek NGeniuS System via the DNA PCR-Free Prep App at different library prep input masses. Concentration of samples was measured using a Qubit ssDNA Assay Kit. Molarity is calculated based on an expected 450 bp average fragment size and 660 g/mol mass per the Illumina reference guide documentation.

The 100 ng and 2000 ng input mass pools were sequenced on a NovaSeq 6000, resulting in yields of 1.32 Tbp and 1.22 Tbp of data, respectively. The 300 ng input mass pool was sequenced on a NovaSeq 6000DX, resulting in a yield of 3.75 Tbp of data. All runs passed QC checks and gave %Q30 Average above 86% with Indexing QC CV less than 22%. The automated Biomek NGeniuS library prep met all of the criteria for Illumina's qualifications for mean fragment insert size, % duplicate marked reads, %Q30 bases, normalized coverage, % autosomal callability, and % exome callability.

Metric	100 ng input		300 ng input		2000 ng input	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
% Index CV	21.28	N/A	21.83	N/A	19.00	N/A
% Mapped Reads (30x)	97.17	0.08	97.14	0.10	96.88	0.11
% Duplicate Marked Reads	7.73	0.37	7.22	0.44	9.08	1.08
Mean Insert Size	348	5	453	7	453	9
% Q30 bases R1	90.86	0.22	92.28	0.15	88.64	0.74
% Q30 bases R2	87.99	0.74	87.44	0.56	85.69	1.03
Normalized coverage at GC regions 20-39%	1.056	0.005	1.014	0.006	1.037	0.006
Normalized coverage at GC regions 60-79%	0.876	0.011	0.991	0.018	0.900	0.000
Average Autosomal Coverage	41.84	8.20	42.99	9.04	100.00	14.58
% Autosome Callability	97.50	0.10	97.72	0.12	97.97	0.07
% Autosome Exosome Callability	98.92	0.05	99.04	0.05	99.13	0.02
Transition/Transversion Ratio	1.99	0.000	1.99	0.005	1.98	0.005

Table 5. NovaSeq sequencing results for Biomek NGeniuS DNA PCR-Free Prep App processing of H. sapiens gDNA at three input masses. For 100 ng input, 8 samples excluding negative control were pooled by mass and sequenced using an S2 flow cell. For 300 ng input, 22 samples excluding two negative controls were pooled by volume and sequenced using an S2 flow cell. For 2000 ng input, 3 samples excluding negative control were pooled by mass and sequenced using an S4 flow cell.

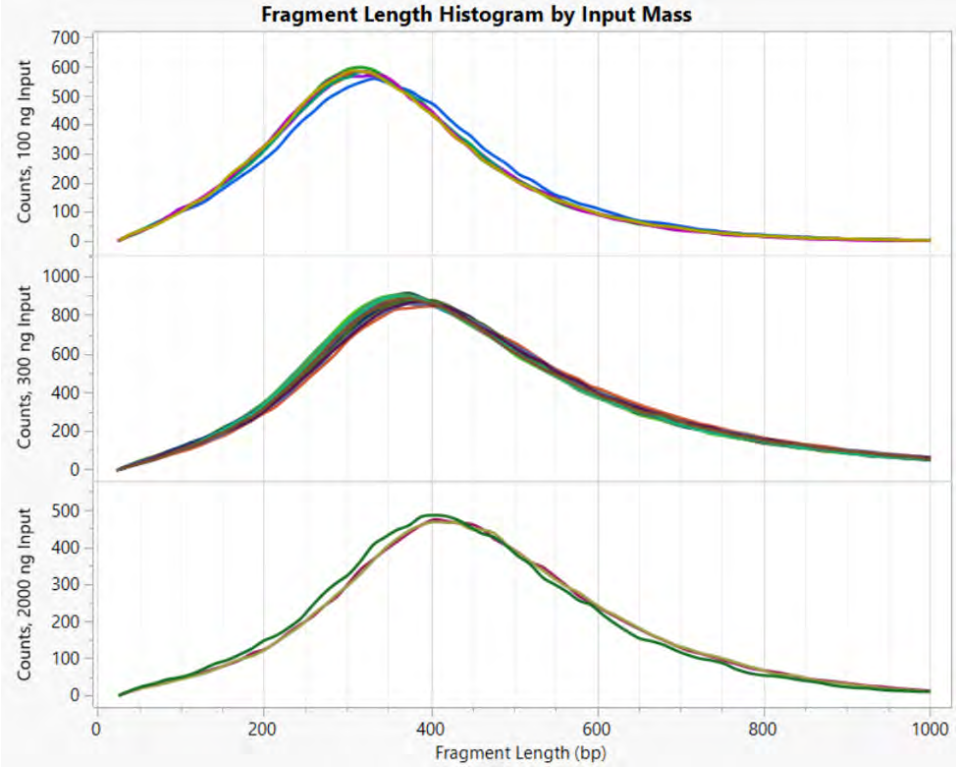
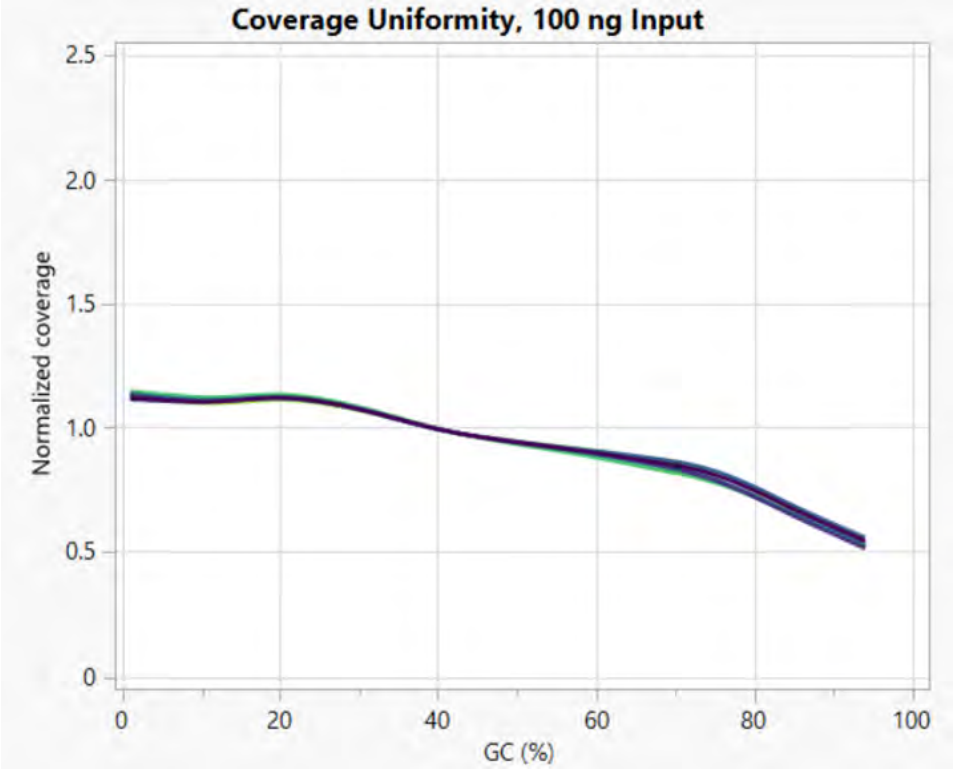
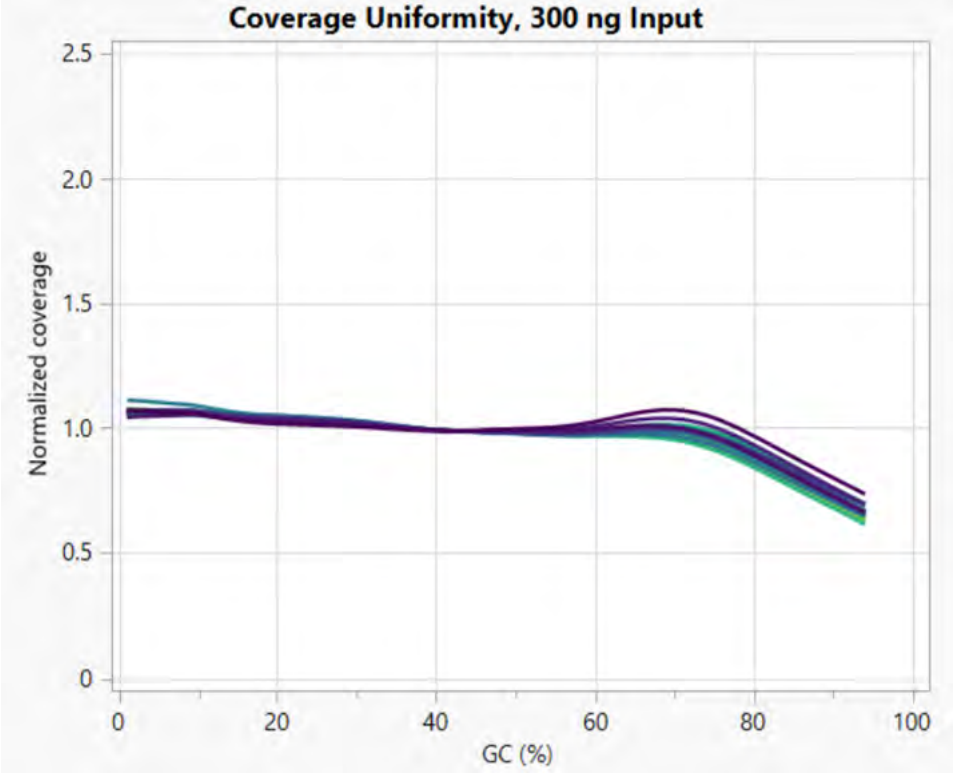


Figure 3. Resulting distribution of library fragment lengths from different input masses. Lower input mass showed a shift towards lower peak fragment length.





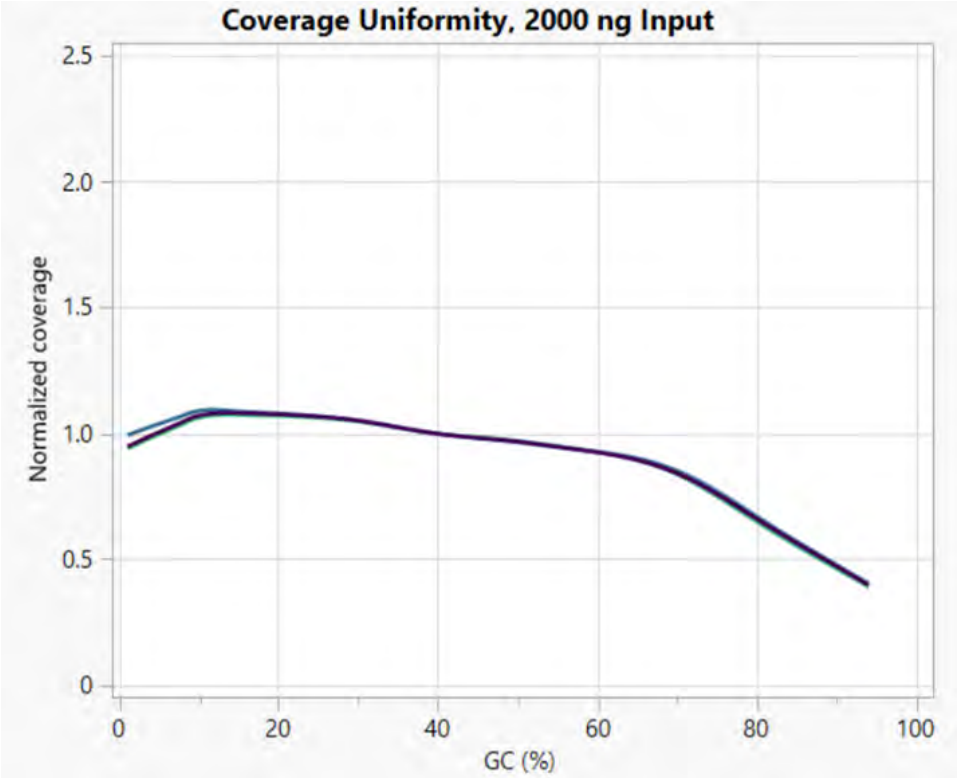


Figure 4. GC-bias plots for the human genome at 100-base windows. Coverage stayed the most even at the protocol-recommended input mass of 300 ng.

Summary:

The Illumina DNA PCR-Free Prep App on the Biomek NGeniuS system produces libraries of sufficient concentration for sequencing across the input range of 100-2000 ng, per the standard protocol. The generated libraries met Illumina's sequencing quality metrics.

References:

1. Fungtammasan A, Ananda G, Hile SE, et al. Accurate typing of short tandem repeats from genome-wide sequencing data and its applications. Genome Res. 2015;25(5):736-749.

2. Illumina DNA PCR-Free Library Prep Reference Guide Document 1000000086922 v03, Feb 2021, **https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/illumina_prep/dna_pcr_free/illumina-dna-pcr-free-reference-guide-1000000086922-03.pdf**

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