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Optimal loading concentrations for Illumina DNA PCR-Free libraries

Load Illumina DNA PCR-Free libraries onto compatible sequencing systems at optimal concentrations for high-quality sequencing data.

Introduction

While Illumina next-generation sequencing (NGS) technology has advanced rapidly in recent years, PCR-dependent library preparation protocols still present significant challenges. PCR bias can lead to uneven coverage across regions of the genome, especially in regions with extreme base composition. To address this concern, Illumina developed Illumina DNA PCR-Free Prep, Tagmentation (Illumina DNA PCR-Free) for library preparation. This advanced solution offers a unique combination of On-Bead Tagmentation with a PCR-free workflow to generate libraries for highly accurate NGS applications, such as tumor–normal variant identification or human whole-genome sequencing (WGS). Library loading concentration is critical for successful sequencing, as it determines cluster density, data output, and data quality. This technical note provides information on optimal loading concentrations for Illumina DNA PCR-Free libraries across compatible Illumina sequencing systems.

Library preparation and optimal loading concentrations

To determine optimal loading concentrations, Illumina DNA PCR-Free libraries were prepared from 300 ng of human reference DNA (Coriell, Catalog no. NA12878) using a thermal cycler standard protocol. With 300–2000 ng genomic DNA (gDNA) input, library yields are normalized, allowing for equimolar pooling by volume of the samples with only a single quantification step after pooling.

After sample multiplexing, all prepared pools were quantified either with the Qubit ssDNA Assay Kit (Thermo Fisher Scientific, Catalog no. Q10212) or the KAPA qPCR Library Quantification Kit (Roche, Catalog no. KK4824). Due to the single-stranded nature of Illumina DNA PCR-Free libraries, size distribution typically seen with NGS libraries cannot be assessed by capillary electrophoresis–based instruments, such as the Bioanalyzer or Fragment Analyzer. Therefore, it is recommended that 450 bp be used as the arbitrary library median insert size and 660 g/mol as the DNA mass for calculating molarity, despite the library being single stranded. The molarity values for ssDNA quantified libraries were calculated using the simplified formula:

$$\text{Molarity (nM)} = \text{yield (ng/µl)} \times 3.36$$

Subsequently, pools were diluted to starting concentrations in resuspension buffer (RSB) (Table 1) and processed according to the Dilute and Denature Libraries Guide specific for each Illumina sequencing system.[1-5] Although libraries are single stranded, they still undergo denaturation to resolve any DNA secondary structures, which can negatively influence sequencing efficiency. Prepared libraries were then sequenced with a paired-end read length of 2 × 150 bp and 10 bp index reads, except for the NextSeq™ 550 System, where the read length was 2 × 149 bp with 10 bp index read.

The Illumina DNA PCR-Free workflow requires the use of custom sequencing primers. The VP10 Custom Read 1 primer is required for all sequencers, while the VP14 Custom Index 2 primer is required for

Table 1: Recommended loading concentrations by sequencing system

Sequencing system	qPCR values–based calculations		ssDNA Qubit values–based calculations		Custom sequencing primer	
	Starting concentration (nM)	Final loading concentration (pM)	Starting concentration (nM)	Final loading concentration (pM)	VP10 Read 1 primer	VP14 Index 2 primer
NovaSeq 6000 standard workflow	1.0-1.5	200-300	2.0-3.0	400-600	Yes	—
NovaSeq 6000 XP workflow	0.75-1	150-200	1.5-2.0	300-400	Yes	—
HiSeq 3000 and HiSeq 4000 Systems	3-3.5	300-350	6-7	600-700	Yes	Yes
HiSeq 2500 Rapid Run mode	2	9-10	4	18-20	Yes	—
HiSeq 2500 High Output mode	2	14-16	4	28-32	Yes	—
NextSeq 500 and 550 Systems	2	1.3-1.4	4	2.6-2.8	Yes	Yes
MiSeq System (v3 reagents)[a]	4	14-16[a]	8	28-32[a]	Yes	—
MiniSeq System	1	1.0-1.1	2	2.0-2.2	Yes	Yes

a. With the MiSeq System, protocol A should be used and 0.2N NaOH should be substituted with 0.1 NaOH.

Table 2: Illumina DNA PCR-Free libraries meet required performance metrics specified by platform

Sequencing system	Cluster density		% Q30		% Occupancy	
	Passing	Illumina DNA PCR-Free	Passing	Illumina DNA PCR-Free	Optimal	Illumina DNA PCR-Free
MiniSeq System	170-220 K/mm²	201-220 K/mm²	> 80%	86-90%	—	—
MiSeq System (v3 reagents)	1200-1400 K/mm²	1223-1281 K/mm²	> 80%	92-94%	—	—
NextSeq 550 System	170-220 K/mm²	178-220 K/mm²	> 75%	84-91%	—	—
HiSeq 2500 Rapid Run Mode	850-1000 K/mm²	980-1018 K/mm²	> 80%	92-96%	—	—
HiSeq 4000 System	—	—	> 75%	76-82%	85-95%	87-88%
NovaSeq 6000 System	—	—	> 75%	87-91%	85-95%	87-93%

HiSeq™ 3000, HiSeq 4000, NextSeq, and MiniSeq™ runs (Table 1). Illumina DNA PCR-Free custom primers are pre-formulated in HT1 buffer and are provided at the final concentration needed for sequencing on any Illumina sequencing platform. To establish optimal loading concentrations for each system, multiple runs were performed at various loading concentrations.

Sequencing metrics with optimal loading concentrations

Illumina DNA PCR-Free libraries sequenced with optimal loading concentrations achieved performance specification metrics on each sequencing system (Table 2). These performance metrics include the following:

- **Cluster density**—a critically important metric for nonpatterned flow cells that influences run quality, reads passing filter, Q30 scores and total data output
- **Q score of 30 (Q30)**—an equivalent of the probability of an incorrect base call 1 in 1000 times, ie, the probability of a correct base call is 99.9%
- **Percent occupancy**—the percentage of clusters on a patterned flow cell that have DNA that can be sequenced

Due to the different mechanism of clustering between nonpatterned (MiniSeq, MiSeq™, NextSeq 500, NextSeq 550, and HiSeq 2500 Systems) and patterned (HiSeq 3000, HiSeq 4000, and NovaSeq™ 6000 Systems) flow cells, variation in the median insert size of sequenced libraries is observed between systems. These size differences are not reflective of library preparation.

While Illumina DNA PCR-Free median insert size on the NovaSeq 6000, HiSeq 3000, and HiSeq 4000 Systems oscillates around 450 bp, when sequenced on lower-throughput systems, the observed median insert size will be reduced (< 400 bp). Degradation of starting gDNA and/or presence of inhibitors in initial material may further influence the insert size median. However, to align with Illumina recommended loading concentrations, the use of 450 bp is required in the library molarity calculations, independent of sequencing system used.

Summary

Illumina DNA PCR-Free combines On-Bead Tagmentation and a rapid workflow to generate high-quality sequencing libraries. Optimizing the loading concentrations for these libraries ensures high-quality results. This technical note provides recommendations for Illumina DNA PCR-Free libraries across Illumina sequencing systems.

Learn more

To learn more about Illumina DNA PCR-Free, visit www.illumina.com/products/by-type/sequencing-kits/library-prep-kits/dna-pcr-free-prep.html

References

1. Illumina (2019). MiniSeq System Denature and Dilute Libraries Guide. Accessed May 11, 2020.
2. Illumina (2019). MiSeq System Denature and Dilute Libraries Guide. Accessed May 11, 2020.
3. Illumina (2019). NextSeq 500 and NextSeq 500 Systems Denature and Dilute Libraries Guide. Accessed May 11, 2020.
4. Illumina (2019). HiSeq Systems Denature and Dilute Libraries Guide. Accessed May 11, 2020.
5. Illumina (2019). NovaSeq 6000 System Denature and Dilute Libraries Guide. Accessed May 11, 2020.

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