Best Practices for Standard and Bead-Based Normalization in Nextera® XT DNA Library Preparation Kits

For libraries above 15 nM concentration, bead-based normalization provides increased convenience and flexibility.

Introduction

Illumina library prep protocols for next-generation sequencing (NGS) include many features designed to increase ease-of-use and reduce total hands-on time. When working with more than 12 Nextera XT DNA libraries (with concentrations over 15 nM), bead-based normalization is a quick and easy way to normalize libraries so that they are evenly represented in the final sequencing data. However, choosing when to perform standard normalization or bead-based normalization can be a challenge. This technical note provides guidance for deciding when to use standard or bead-based normalization and also provides data from a study comparing the two normalization methods.

Standard Normalization

With any NGS library prep protocol, standard normalization is an important process to understand and is considered a best practice for ensuring optimal clustering and high-quality data. Standard normalization involves quantifying libraries with Qubit, Bioanalyzer, or Fragment Analyzer Instruments, performing calculations for each library (typically in a spreadsheet), and manually diluting the libraries one at a time to a common, or "normalized," concentration. Standard normalization takes place in the library preparation protocol after the amplification and library clean-up steps (Figure 1). While standard normalization can take longer than bead-based normalization, it is the preferred method when:

- 12 libraries or less require normalization
- Library yields are less than 15 nM
- Library yields are highly variable and unpredictable
- Users are working with uncharacterized genomes
- Users are inexperienced with the Nextera XT DNA Library Prep Kit protocol

When the DNA samples used in the library preparation have not been fully characterized in terms of consistent quality and performance, and the library preparation protocol itself is not consistent, there is a risk that library quality and yield will not be appropriate for bead-based normalization. In these cases, it is best to perform standard normalization until the DNA samples and library prep protocol are consistently producing 15 nM concentrations or higher.

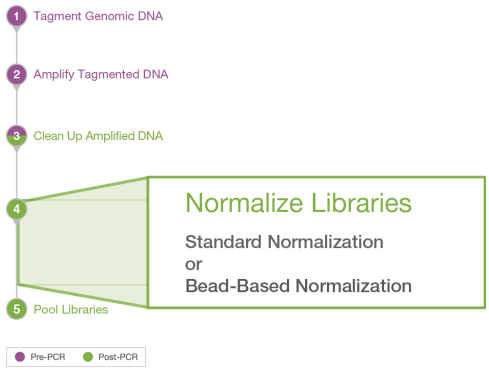

Figure 1: Nextera XT DNA Library Prep Workflow—Standard or bead-based normalization steps occur after library amplification and clean-up steps.

Bead-Based Normalization

In addition to standard normalization, the Nextera XT DNA Library Prep Kit offers bead-based normalization as an efficient, time-saving alternative. During bead-based normalization, DNA is bound to normalization beads and eluted off the beads at approximately the same concentration for each sample. Bead-based normalization enables scientists to bypass time-consuming library quantitation measurements and manual pipetting steps before loading libraries onto the sequencer. Bead-based normalization can provide significant cost and time savings for researchers processing many samples, or for researchers without access to Bioanalyzer or Fragment Analyzer instruments. Bead-based normalization is the preferred method when:

- 12 libraries or more require normalization
- Library yields are more than 15 nM
- Library yields are consistent and predictable
- Users are working with well-characterized genomes
- Users are experienced with the Nextera XT DNA Library Prep Kit protocol

All the reagents needed to perform bead-based normalization are included in Nextera XT DNA Library Prep Kit. To view or download the Nextera XT DNA Library Prep protocol, or any other Illumina library preparation protocols, visit the Sequencing Library Prep Protocols page.

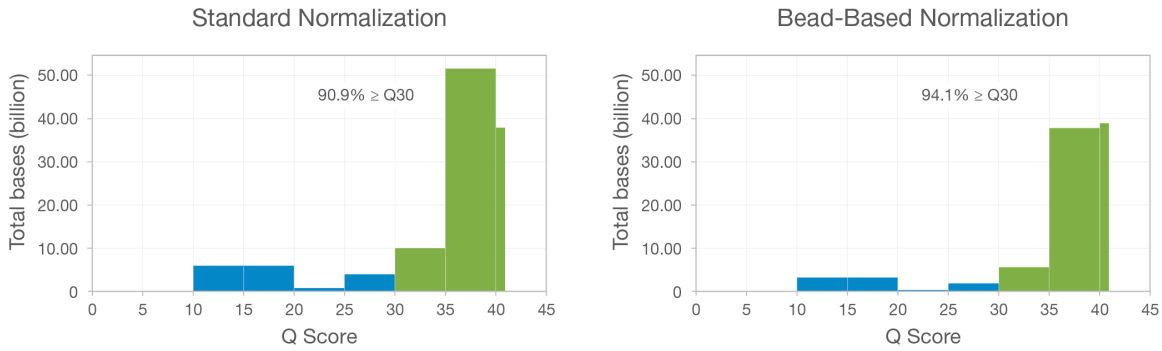

Figure 2: Comparison of Library Q30 Scores—The percentage of bases scoring above Q30 were analyzed and displayed in BaseSpace® Sequence Hub. Libraries prepared with standard and bead-based normalization produced data with over 90% Q30 scores. In fact, the majority of bases demonstrated above Q35 scores, indicating that both normalization methods produce exceptional data quality.

Materials and Methods

This technical note presents a direct comparison of standard normalization and bead-based normalization methods using the Nextera XT DNA Library Preparation Kit. The results demonstrated high data quality for both methods, as well as significant workflow advantages with bead-based normalization.

Library Preparation

To compare library normalization methods, 96 libraries were prepared with the Nextera XT DNA Library Preparation Kit (Illumina, Cat No. FC-131-1096) (Figure 1). The libraries were prepared with 32 *Escherichia coli* MG1655 samples (American Type Culture Collection (ATCC), Cat No. 700926D-5), 32 *Bacillus cereus* samples (ATCC, Cat No. 10987D-5), and 32 *Rhodobacter sphaeroides* samples (ATCC, Cat No. 17023D-5). These strains were chosen because their genomes contain a range of GC content: *E. coli* contains 51%, *B. cereus* contains 35%, and *R. sphaeroides* contains 69% GC content. After the amplification and clean-up steps, each library was divided for standard and bead-based normalization.

Library Normalization

Libraries prepared with standard normalization were quality checked for fragment size and fragment uniformity on the Fragment Analyzer (Advanced Analytical Technologies) using the High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical Technologies, Cat No. DNF-474). Libraries can also be qualified with the 2100 Bioanalyzer (Agilent Technologies) using a High Sensitivity DNA Kit (Agilent Technologies, Cat No. 5067-4626). Libraries prepared with standard normalization methods were quantified with the Qubit dsDNA HS Assay Kit (Invitrogen, Cat No. Q32854).

For bead-based normalized libraries, the final library pool consists of single-stranded DNA, which does not resolve well on an agarose gel or Bioanalyzer chip.

Standard Normalization Calculations

1. After running the libraries on the Qubit and Fragment Analyzer, the following formula was used to calculate the nM concentration of each library:

   $nM = ((ng/ul)/ (avg\ bp\ size \times 660\ g/mol))^* 1{,}000{,}000$

2. The libraries were then normalized to 2 nM each, using:

   $C_i \times V_i = C_f \times V_f$,

   then $V_f - V_i$ = µl resuspension buffer (RSB) to add

3. Example:

   $V_f = (15\ nM \times 2\ µl)/2\ nM$

   $V_f = 15\ µl$

   µl RSB = 15 µl -2 µl

   µl RSB = 13 µl

4. In the final step, 5 µl from each 2 nM library were pooled together in 1 tube for sequencing.

Sequencing and Preliminary Data Analysis

Libraries prepared with standard normalization were loaded onto the HiSeq® 2500, in Rapid Run mode, with HiSeq Rapid SBS Kit v2 sequencing reagents (Illumina, Cat No. FC-402-4023) at 8.5 pM. The bead-based normalized libraries were loaded onto the HiSeq 2500, in Rapid Run mode, with HiSeq Rapid SBS Kit v2 sequencing reagents, following the HiSeq and GAIIx Systems Denaturing and Dilute Libraries Guide guidelines of 15 µl library volume.

Results

Comparison of Sequencing Data Quality

Libraries normalized with standard and bead-based methods produced comparable, high-quality data, with >94% reads passing filter (Table 1) and >90% of reads ≥ Q30 (Figure 2).

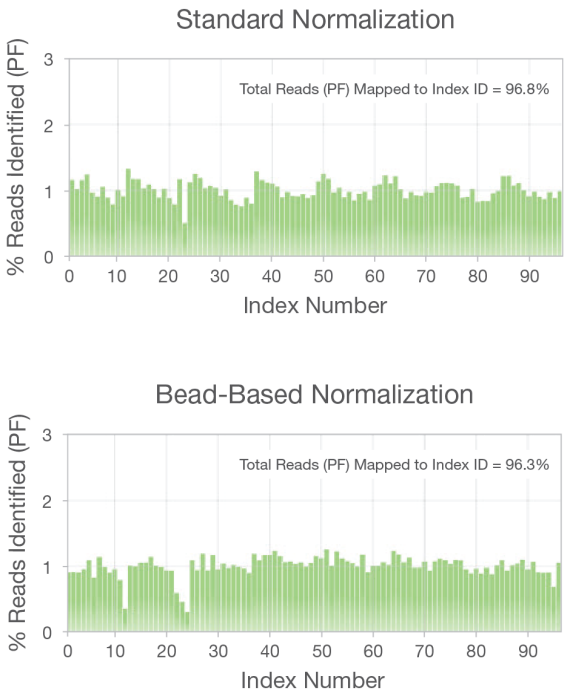

Figure 3: Comparison of Reads Mapping to Indexes—The index charts generated and displayed in BaseSpace Sequence Hub show the percentage of Pass Filter (PF) reads that mapped to each of the 96 indexes (eg, 96 libraries). The index charts demonstrate that the individual libraries are evenly represented in the normalized pooled samples and that this distribution of libraries is similar between the standard and bead-based normalized libraries. Four libraries in the bead-based normalized pool (index numbers 12 and 22–24) displayed a lower percentage of reads compared to other libraries in the pool. These four libraries had lower than 15 nM concentrations before the bead-based normalization steps.

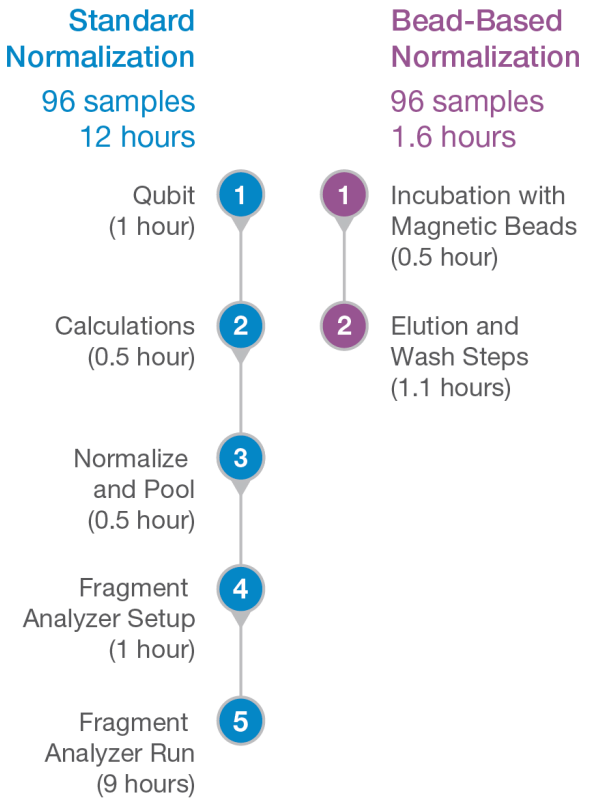

Figure 4: Comparison of Normalization Workflow Times—Standard normalization took 12 hours total, while bead-based normalization took 1.6 hours total. Note: Fragment Analyzer times vary depending on instrument configuration (eg, 12 sample, 48 sample, or 96 sample capillary heads are available). For this study, a 12 sample capillary head was used.

Table 1: Standard and Bead-Based Normalization Sequencing Data Quality

	Standard Normalization	Bead-Based Normalization
%PF Reads	94.1 %	97.0 %
% ≥ Q30	90.9 %	94.1 %

Comparison of Indexing Performance

Both sets of 96 libraries demonstrated a high percentage of reads mapped to Index IDs, indicating successful indexing during library preparation (Figure 3). Furthermore, both sets of libraries demonstrated an equal distribution of individual libraries within the pooled, normalized sample (Figure 3).

Comparison of Workflow Times

To compare workflow time requirements, the steps involved in standard normalization and bead-based normalization were timed and recorded. The standard normalization workflow took three hours of hands-on time for Qubit quantitation, normalization calculations, normalization of libraries with manual pipetting, and Fragment Analyzer instrument setup (Figure 1). The Fragment Analyzer run took 9 hours, contributing to a total protocol time of 12 hours. The bead-based normalization method was significantly faster with a total hands-on/total protocol time of 1.6 hours.

Cost-Savings with Bead-Based Normalization

Beyond providing a faster workflow, bead-based normalization delivers significant cost savings per sample. Because standard normalization requires the purchase of additional equipment and kits (Table 2), bead-based normalization provides savings up to $500 per set of 96 samples compared to standard normalization .

Table 2: Additional Kits Required for Standard Normalization

	Vendor	Catalog No.
High Sensitivity NGS Fragment Analysis Kit	Advanced Analytical Technologies	DNF-474
High Sensitivity DNA Kit	Agilent Technologies	5067-4626
Qubit dsDNA HS Assay Kit	Invitrogen	Q32854

Fragment size analysis can be performed with either the High Sensitivity NGS Fragment Analysis Kit or the High Sensitivity DNA Kit.

Summary

The Nextera XT DNA Library Prep Kit protocol offers standard and bead-based normalization for different library preparation needs. Both methods produce high-quality libraries, with excellent clustering, pass-filter, and Q30 scores. For high-throughput researchers, bead-based normalization is an option that saves time and resources by providing an accelerated path from DNA to data.

Illumina, Inc. • 1.800.809.4566 toll-free (US) • +1.858.202.4566 tel • techsupport@illumina.com • www.illumina.com