The xGen™ Normalase™ Module combined with an xGen Library Prep Kit delivers a streamlined direct sequencing workflow

Abstract

Some of the most widely-used direct sequencing applications include whole genome sequencing (WGS), whole transcriptome sequencing, and shotgun metagenomics to investigate the composition of complex samples. Often these studies have a high volume of samples with specific sequence depth requirements, where data quality, cost, and turnaround time are important factors in choosing a workflow. The xGen Normalase Module combined with one of the available xGen Library Prep Kits is an efficient solution for multiplexing applications. Normalase is an enzymatic library normalization chemistry that provides a balanced multiplexed sequencing pool. The process streamlines library normalization by allowing simple equal volume pooling, eliminating individually quantifying samples that may have different pooling volumes. Combining the xGen Normalase Module with an xGen Library Prep Kit delivers a streamlined workflow for analyzing uniform sample read depth, resulting from fewer discarded reads when downsampling. The Normalase workflow is suitable for various sample types and library preps by providing a uniform multiplexed sequencing pool with a coefficient of variation (CV) of less than 11%. The data quality across a broad base composition range shows the advantages of streamlining the library normalization steps before multiplexed sequencing.

Introduction

Combining the xGen Normalase Module with an xGen Library Prep Kit is suitable for various sample types and library prep workflows for reproducible performance. The xGen DNA Library Prep Kits offer unique library prep strategies for enzymatic and mechanical fragmentation-based library prep. The xGen DNA Library Prep EZ kits use enzymatic DNA fragmentation, while the xGen DNA Library Prep MC kits are compatible with Covaris® sheared DNA or samples that do not require fragmentation. The xGen DNA Library Prep Kits EZ and MC offer easily automated, two-hour workflows with only two enzymatic steps plus PCR amplification (see Figures 1 and 2). The kits result in high complexity libraries that support a broad GC composition with minimal bias. Both kits offer an indexing by PCR workflow using stubby Y adapters during ligation and indexing primers during library amplification. The xGen DNA Library UNI kits offer an indexing by ligation workflow using indexed, full-length adapters during ligation and P5/P7 primers during library amplification. When combining one of the xGen Library Prep Kits with the xGen Normalase Module, library amplification using Normalase conditioning primers is required. The xGen Normalase Module includes xGen Normalase P5/P7 primers. However, xGen Normalase indexing primers are also provided separately and are available in single-use 96-well plates with premixed UDIs up to 1536-plex sequencing, or as CDIs for 96-plex sequencing.

The xGen RNA Library Prep Kit and xGen Broad-Range RNA Library Prep Kit also offer efficient workflows using proprietary Adaptase™ chemistry to construct libraries from first-strand cDNA without requiring second-strand synthesis and degradation steps to maintain strandedness. The xGen RNA Library Prep Kit has a more streamlined 3.5-hour workflow, whereas the xGen Broad-Range Library Prep Kit supports lower input quantities down to 10 ng total RNA. Both produce high-complexity transcriptome libraries without the requirement for adapter titration, which enables a simple workflow across the supported input range while maintaining ligation efficiency at low input. Both kits are compatible with upstream mRNA selection and ribodepletion modules, and both kits offer an indexing by PCR workflow. When combined with Normalase, library amplification using Normalase conditioning primers is required. Normalase indexing primers are supplied separately and are available in single-use 96 well plates with premixed UDIs up to 1536-plex sequencing, or as CDIs for 96-plex sequencing.

Combining the xGen Normalase Module with an xGen Library Prep Kit is an efficient solution to support multiplex applications. Normalase is an enzymatic library normalization chemistry that enables more balanced multiplexed sequencing pools with a <11% CV, which simplifies pooling by quantitative PCR (qPCR) and fluorometric quantification methods. The process also streamlines library normalization since individual sample quantification and different per sample pooling volumes becomes unnecessary. The streamlined workflow, combined with more uniform sample read depth, saves time and results in fewer discarded reads when downsampling. The xGen Normalase workflow also reduces the number of samples that do not meet a required minimum sequence depth threshold, preserving data and lowering sequencing cost. The xGen Normalase technology is compatible with xGen Library Prep Kits, including the xGen DNA Library Prep Kit for enzymatic or mechanical fragmentation, as well as the xGen RNA Library Prep Kit and the xGen Broad-Range RNA Library Prep Kit. For best results, use the xGen Normalase workflow for sample types and library prep workflows with reproducible performance and routine samples that produce consistent results.

In this application note, we demonstrate performance of the xGen Normalase workflow for three common direct sequencing applications: whole genome sequencing, shotgun metagenomic sequencing, and RNA-seq. Regardless of the application, experimental results show uniform multiplexed sequencing pools with a CV <11% without impact to data quality across a broad range of base compositions. In summary, the xGen Normalase Module streamlines the library normalization steps for multiplexed sequencing to support your next discovery.

Methods

For **enzymatic fragmentation**, 100 ng of Coriell NA12878 DNA (*n* = 24) was used to create a library using the xGen DNA Library Prep Kit EZ according to the recommendations in the xGen DNA Library Prep EZ Kit protocol with a target insert size of 200 bp. Ligation of xGen Stubby Y adapters (reagent W5) was followed by indexing PCR with xGen Normalase UDI Indexing Primers for three amplification cycles. The non-Normalase libraries (*n* =12) were quantified by qPCR and the fragment size was analyzed by a Bioanalyzer® (Agilent) before being pooled into a 4 nM multiplexed library. Normalase libraries (*n* = 12) were pooled into a multiplexed 4 nM library according to the recommendations in the xGen Normalase Module protocol. The qPCR (non-Normalase) pool and the Normalase pool were combined by equal volume to generate a final 4 nM pool containing all the 24 library preps. The final pool (12 pM) was loaded on a MiSeq™ (Illumina) 300-cycle kit (2 x 101 bp read length and 2 x 10 bp index reads).

For **mechanical fragmentation**, 100 ng of Coriell NA12878 DNA (*n* = 24) was converted into a NGS library using the xGen DNA Library Prep MC UNI Kit according to the recommendations in the xGen DNA Library Prep MC UNI Kit protocol. DNA was fragmented to a 200 bp insert size using ultrasonication (Covaris instrument). The adapter ligation step was by performed using full-length xGen UDI-UMI adapters and the xGen Normalase Reagent R5 for three cycles of PCR. Non-Normalase libraries (*n* = 12) were quantified by qPCR and the fragment size was determined using a Bioanalyzer (Agilent) before being pooled to a 4 nM equimolar multiplexed sample. Normalase libraries (*n* =12) were generated and pooled to a separate equimolar 4 nM multiplexed pool. The qPCR and Normalase pools were then combined by equal volume to generate a final 4 nM pool for sequencing. Final pool (12 pM) was loaded on a MiSeq 300-cycle kit (2 x 101 bp read length and 2 x 8 bp index reads).

For **metagenomic sequencing**, libraries (*n* = 4) from 1 ng inputs of ATCC® MSA-1000 DNA containing ten bacterial genomes were prepared using the xGen DNA Library Prep EZ Kit using the recommendations in the xGen DNA Library Prep EZ protocol with a target insert size of 350 bp. Adapter ligation was performed using the xGen Stubby Y-adapter (reagent W5) and indexing PCR for CDIs attachment was performed for ten amplification cycles. Samples were quantified by Qubit® (Thermo Fisher Scientific) and the fragment size was determined by Bioanalyzer and then pooled to a 4 nM multiplex library. The final pool (1.8 pM) was loaded on a NextSeq™ (Illumina) 550 300-cycle kit (2 x 151 read length and 2 x 8 bp index reads).

RNA-seq libraries from Universal Human Reference RNA (Thermo Fisher Scientific; Cat. No. QS0639) underwent ribodepletion using Ribo-Zero™ Plus rRNA Depletion Kit (Illumina, Cat. No. 20037135) or poly(A) selection using NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Cat. No. E7490L) prior to library preparation using the xGen RNA Library Prep Kit (Cat. No. 10009814) or xGen Broad-Range RNA (Cat. No. 10009813) Library Prep Kit in either duplicate or triplicate. Indexing PCR using xGen Normalase CDI primers and appropriate number of PCR cycles per input recommendations provided in the respective protocols were performed. Library pools were normalized using the xGen Normalase Module, followed by co-sequencing to determine percent reads identified for each index—either a MiniSeq™ High Output (2 x 76) run, or a NextSeq™ 2000 P3 flow cell (2 x 76) run.

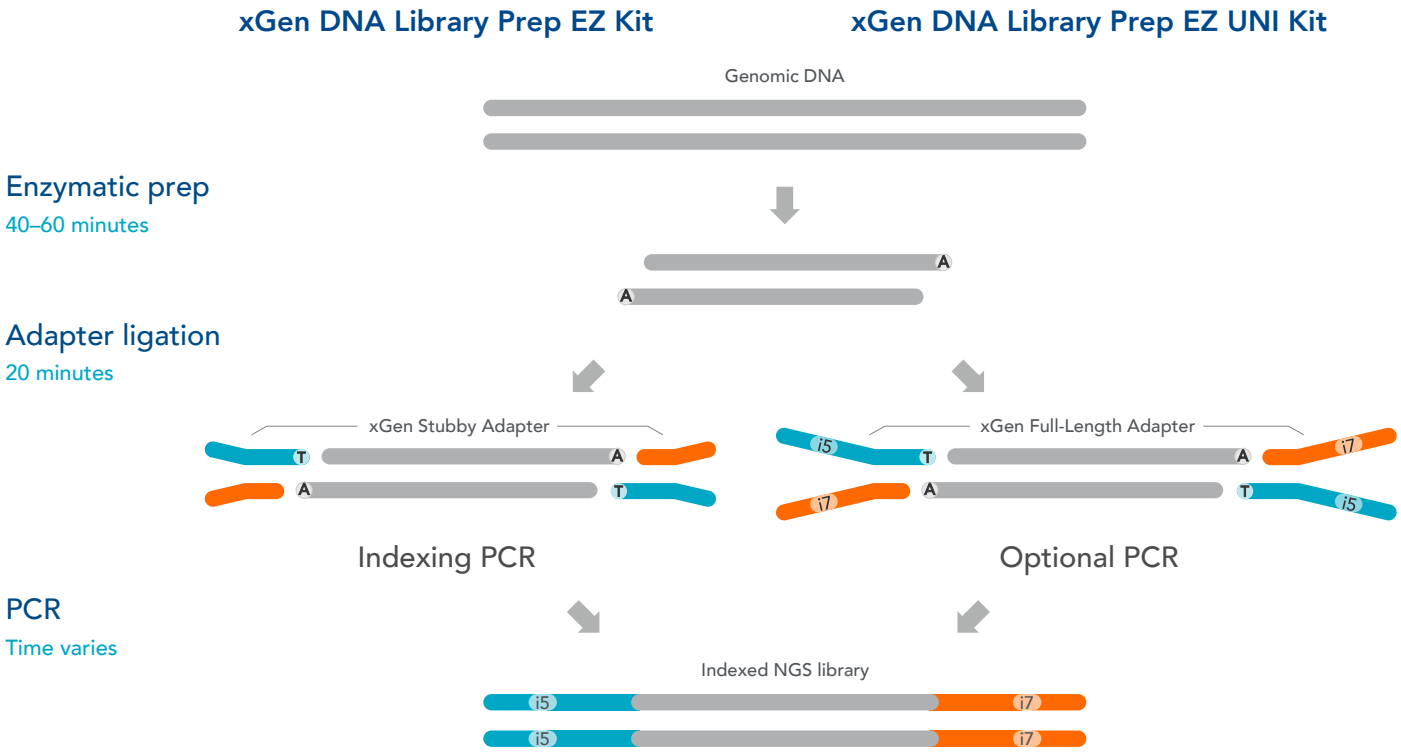

Figure 1A. Workflow for the xGen DNA Library Prep EZ Kits. The xGen DNA Library Prep EZ Kits are compatible with enzymatic fragmentation and use either an indexing by PCR workflow using xGen Stubby Adapters included in the kit and indexing primers supplied separately (left) or an indexing by ligation workflow using full length, indexed Y adapters (right). Both kits include amplification reagents for Indexing PCR and library amplification. xGen DNA Library Prep EZ Kits are compatible with the Normalase module. The full-length indexed Y adapters options are available, including UDI/UMI adapters combined with Normalase primers, and indexing primers with up to 1536-plex Normalase UDI primer pairs.

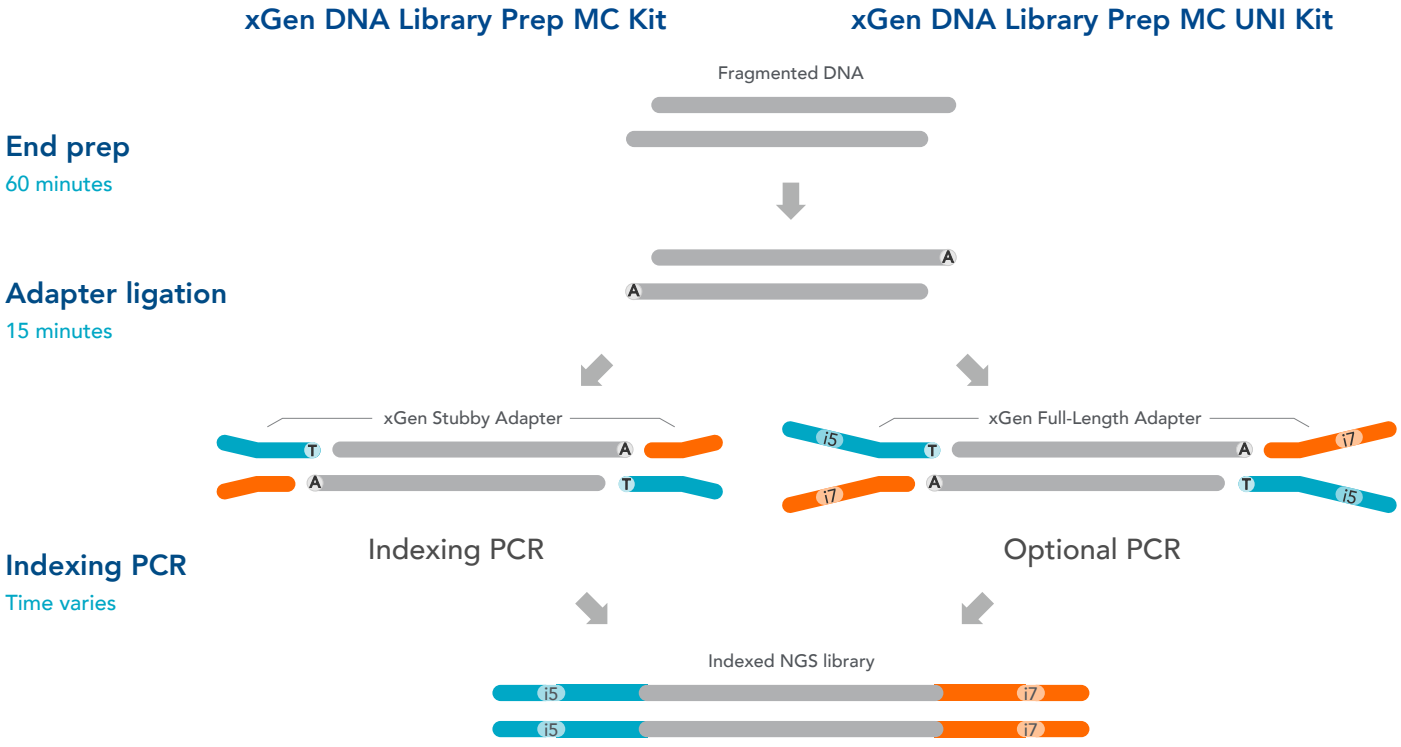

Figure 1B. Workflow for the xGen DNA Library Prep MC Kits These kits are compatible with mechanically sheared DNA, and available in two configurations to support different indexing workflows. The incubation steps consist of end repair, polishing of dsDNA, and A-tailing, all performed in a single End Prep reaction followed by ligation of either a stubby Y adapter (xGen DNA Library Prep MC kit, left) or full-length indexed Y adapter (xGen DNA Library Prep MC UNI Kit, right). The xGen DNA Library Prep MC workflow incorporates an indexing PCR step after adapter ligation to complete the adapter sequences. The xGen DNA Library Prep MC Kits are compatible with the Normalase module and various indexing primers and full-length indexed Y adapters, including UDI / UMI adapters followed by Normalase PCR and Normalase indexing primers up to 1536-plex UDI primer pairs.

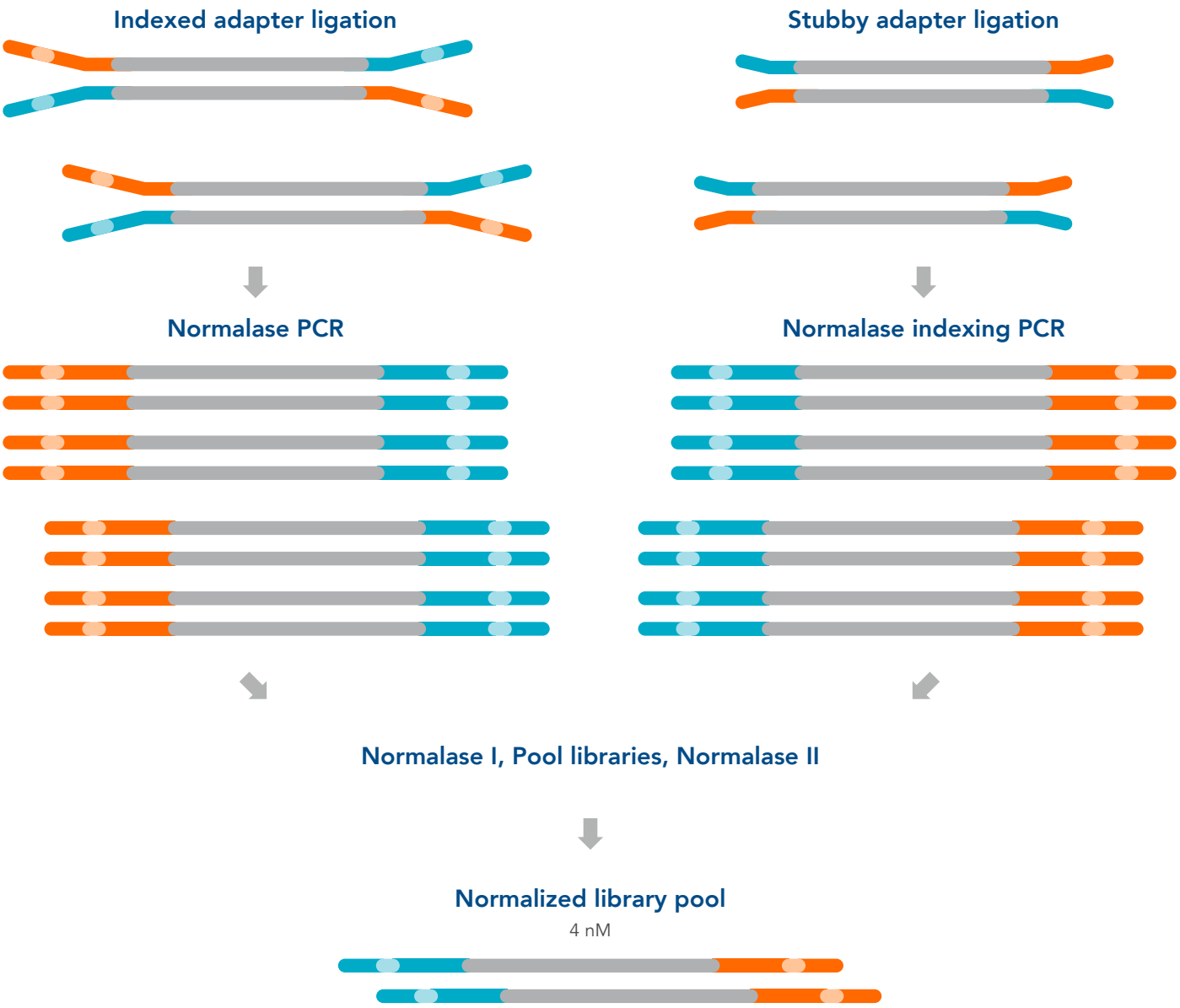

Figure 2. Normalase workflow. The xGen Normalase workflow begins after NGS library adapter ligation, using either full-length indexed adapters or truncated adapters. Then Normalase PCR primers are used to amplify the libraries to above the minimum threshold and condition the libraries for downstream Normalase enzymology. For full-length indexed adapter libraries, xGen Normalase terminal primers are used; for stubby adapter ligated libraries, xGen Indexing Normalase primers are used. The result of the Normalase workflow is a balanced, multiplexed NGS library pool that is ready for sequencing.

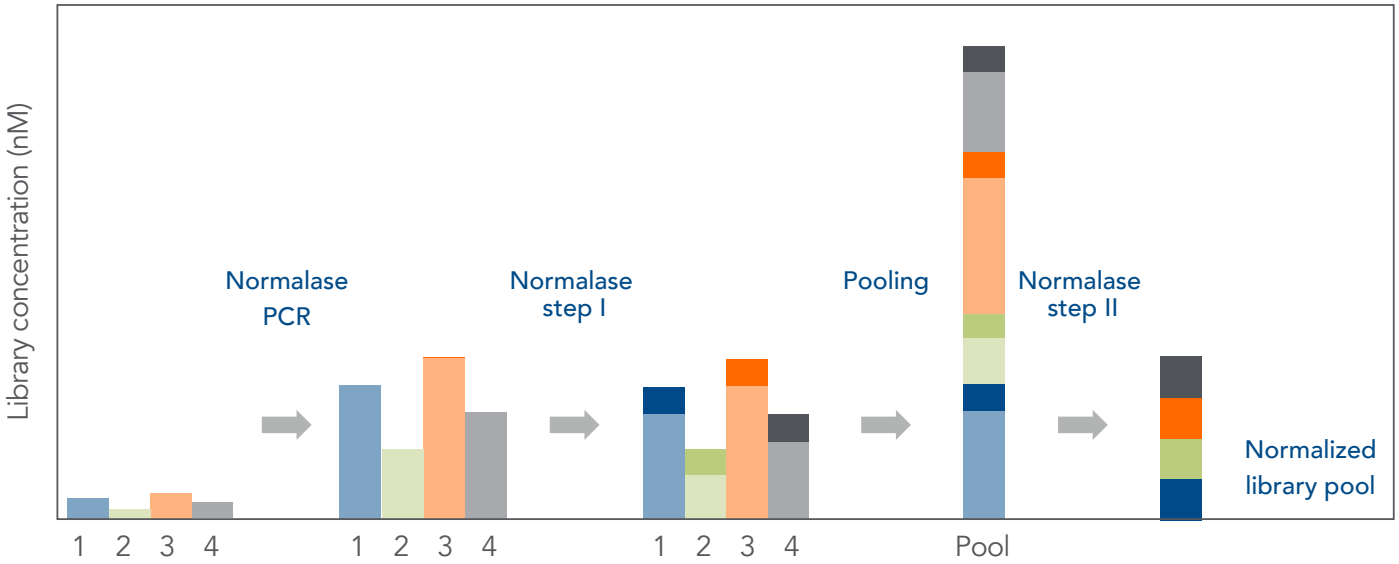

Figure 3. xGen Normalase Module generates an equimolar library pool for multiplexing. Normalized libraries are produced in four main steps. First, Normalase PCR increases the initial library concentrations. Second, Normalase I treatment selects a 4 nM library fraction. Third, the samples are pooled in equal amounts for multiplexed sequencing. Lastly, the Normalase II step generates the final pool of libraries. Four libaries following adapter ligation are shown on the first step through the third, where Normalase PCR produces ≥ 12 nM library yields using Normalase primers. Then, the Normalase I step occurs, represented by the 4 nM selected fraction that is shaded darker at the top of the bars above). Finally, equal volume pooling and the Normalase II step produce an equal molar (normalized) library pool.

Results

Whole genome DNA libraries were prepared using the xGen DNA Library Kit EZ and xGen DNA Library Kit MC (Figure 1A and 1B) and the final libraries were pooled and normalized (Figure 2 and 3). A Normalase treated library pool and a library pool quantified by qPCR for conventional library normalization were co-sequenced to determine the read depth variation obtained for each method (Figure 4). A lower coefficient of variation (CV) was observed for the Normalase pools compared to qPCR-based normalization (Figure 4). This indicates that the Normalase pool has a more uniform depth, reducing the number of lost reads when downsampling. The qPCR-based normalization resulted in low depth and did not meet the minimum depth threshold for sequencing. These results demonstrate that the Normalase Module provides an effective WGS solution.

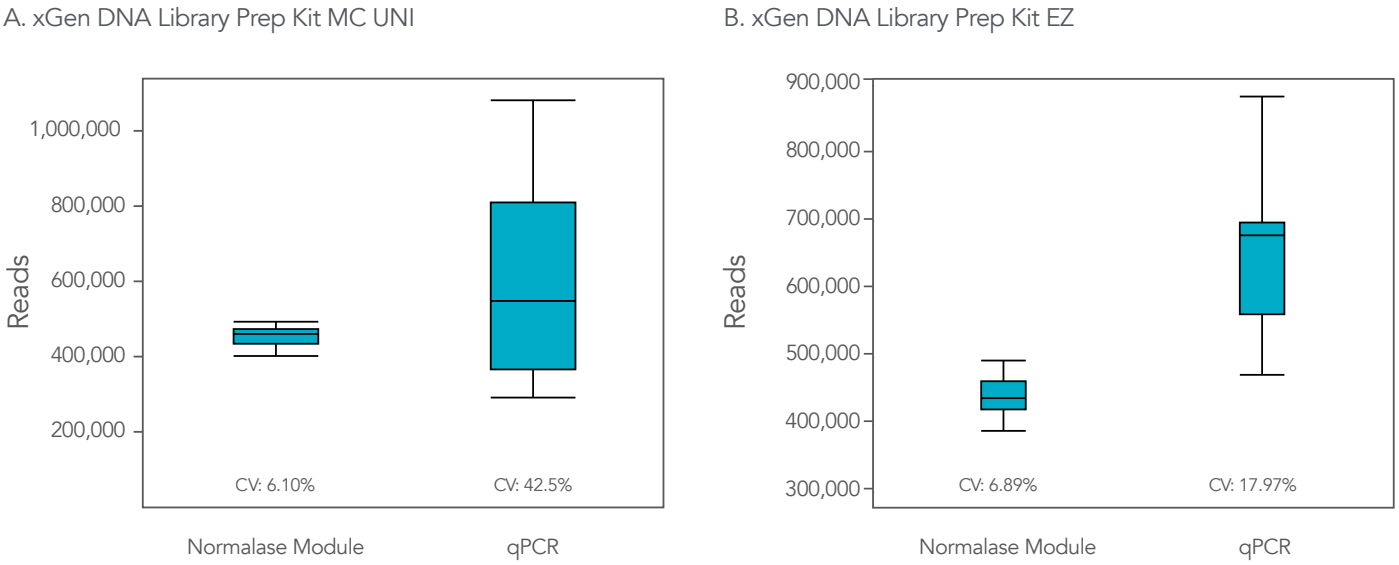

Figure 4. Normalase enables streamlined library balancing and pooling without the need to quantify samples, while maximizing sequencing efficiency. (**A**) xGen DNA Library Prep EZ libraries were generated with stubby adapters, while (**B**) xGen DNA Library Prep MC UNI libraries were generated with xGen UDI-UMI adapters (*n* = 24 each) from 100 ng of NA12878 gDNA. Twelve library subsets were either pooled and normalized based on qPCR quantification, or pooled and normalized using the xGen Normalase Module, followed by co-sequencing to determine the percent reads identified for each index (MiSeq V2 Standard 300 cycle).

For the shotgun metagenomic sequencing workflow, libraries were constructed using DNA derived from a mock metagenome DNA standard with varying GC content before being sequenced. The data shows that Normalase pools maintained the desired insert size of 350 bp across a wide GC range (30–70%) (Figure 5). The Normalase library pools have consistent insert sizes (shown in blue) across the range of GC content (shown in gray). This demonstrates that the libraries pooled using the Normalase workflow maintain a high-quality sequencing coverage across a range of GC targets.

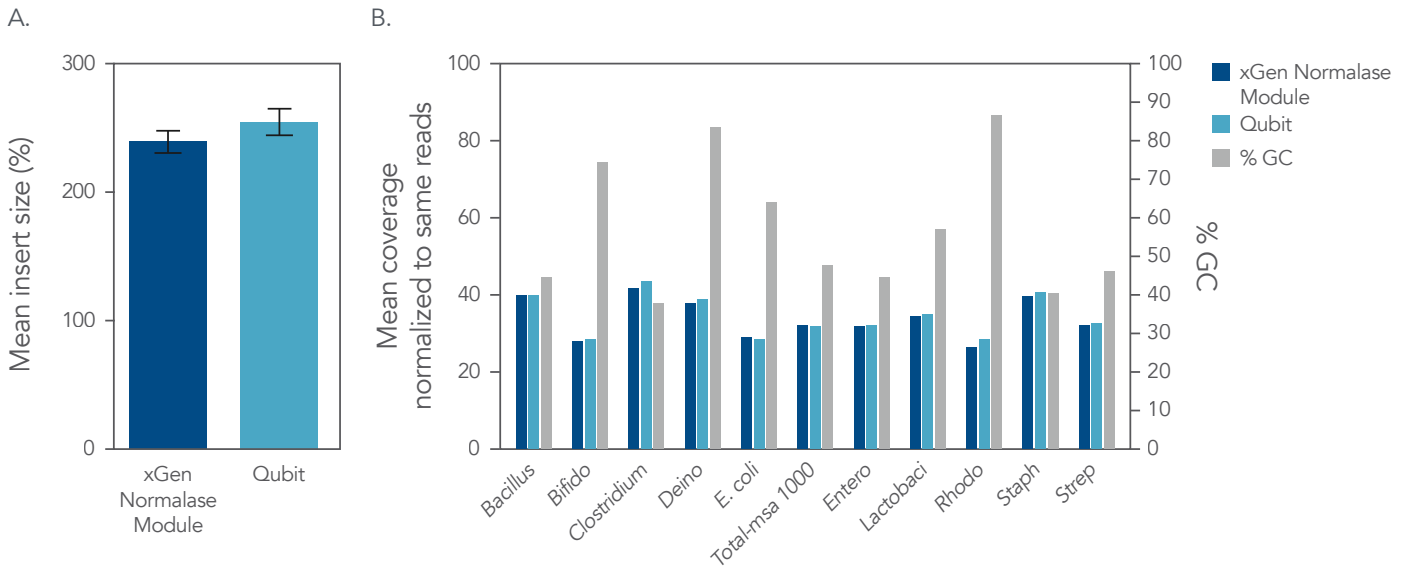

Figure 5. Insert size and coverage. xGen DNA libraries (*n* = 4) were made from 1 ng inputs of ATCC MSA-1000 DNA—an equal mass mix of 10 different bacterial strain genomes with varying GC%. Two libraries were either amplified and indexed with xGen CDI Normalase primers or IDT CDI primers. Normalase conditioned libraries were normalized to 4 nM and pooled using Normalase, while standard libraries were Qubit™ (Thermo Fisher Scientific) quantified and pooled at 4 nM. Libraries were co-sequenced on a MiniSeq (Illumina) at 2 x 150 bp read length using High Output reagents. Across bacterial genomes with varying GC% libraries, Normalase-treated libraries-maintained (**A**) insert size and, (**B**) high-quality genomic coverage.

The strategy of high-throughput pooling RNA-seq libraries in gene expression studies has been shown to have the potential to lower the cost of the data generation process, as well as optimize the statistical power for gene expression. Since the cost of RNA sample preparation is relatively low, one may consider pooling as many RNA samples as possible to capture the heterogeneity of the population under study. However, errors in manual library pooling and normalization can cause deficient read distribution across indexed libraries within a pool and that could have a significant impact on direct comparison of mRNA expression measures.

The coefficient of variation (CV) for RNA-seq library pools created using xGen Normalase technology showed a uniform sequencing depth, which was independent of the number of indexed libraries within a pool, or the type of sequencer used (Figure 6). The data showcases the use of Normalase in providing a cost-effective workflow for high-throughput RNA-seq library normalization and pooling, with optimal index balancing for reliable quantitative differential expression analyses.

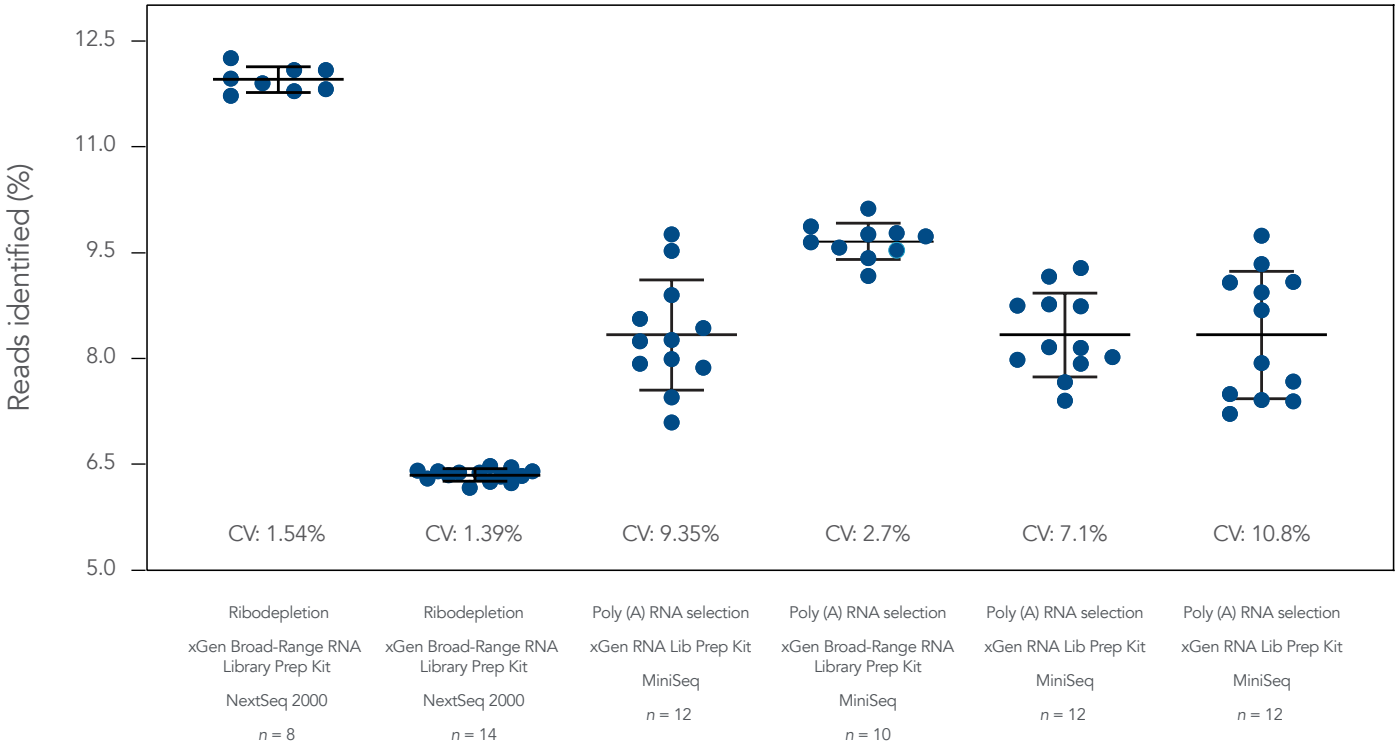

Figure 6. Normalase enables consistent pooling of RNA-seq libraries without compromising transcriptomic data quality. xGen RNA and xGen Broad Range RNA libraries using universal human total RNA samples (Thermo Fisher Scientific; Cat. No. QS0639) were either ribodepleted or poly(A) selected upstream of library construction. Libraries were indexed using xGen Normalase CDI primers and amplified with the number of PCR cycles provided in the respective protocols. Library pools were then normalized using the Normalase workflow and sequenced to determine the percent reads identified for each index. Respective sequencing platforms are indicated in the figure.

Conclusions

The xGen DNA Library Prep Kits are DNA library preparation workflows for direct sequencing applications including WGS, shotgun metagenomics, and RNA-seq. Combined with xGen DNA Library Prep Kits, the xGen Normalase Module enables a streamlined library balancing and pooling process that eliminates the need to perform sample quantification individually, maximizing sequencing efficiency.

The xGen™ Normalase™ Module combined with an xGen Library Prep Kit delivers a streamlined direct sequencing workflow

Technical support: applicationsupport@idtdna.com