Streamlined target enrichment and uniform sequencing using xGen™ Pre-Hybridization Capture Normalase™ technology

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Introduction

In traditional hybrid capture workflows, researchers are required to spend valuable time performing quality control on each library and individually pooling varying volumes to create multi-plex pools for capture. This method is time-consuming, costly, and prone to pooling and pipetting errors. To circumvent these challenges, we have developed a novel, enzymatic library normalization technology that eliminates the need for library quantification and uses equal volume pooling prior to hybridization capture resulting in a streamlined workflow with a sample read depth CV of ≤10%. The thoughtfully developed normalization module can be integrated seamlessly into several xGen DNA and RNA Library prep workflows including the xGen cfDNA & FFPE and xGen EZ library prep kits. Here we show how the xGen Pre-Hybridization Capture Normalase Module can save time and money by outperforming alternative methods such as bead-based normalization and Qubit dsDNA quantification, while enabling high-throughput sample processing with the ability to normalize different library input amounts, and results in expected variant allele frequencies, ensuring that the normalization process does not interfere with downstream applications.

xGen Pre-Hybridization Normalase Technology reduces hands on time and streamlines NGS library quantification

xGen Pre-Hybridization Capture Normalase Module:

- Seamlessly integrates into existing library preparation workflows and reduces hands-on-time and total workflow time compared to fluorometric and qPCR methods (**Fig. 1**)
- Generates equimolar library pool for downstream hybrid capture (**Fig. 2**)
- Optimal for use with library prep workflows and sample types that produce consistent library yields
- Selects a broad range of library inputs into hybridization capture (100 - 500 ng), supports multiple insert sizes (150 – 350 bp), and multiplexing of 4 – 24 libraries per pool

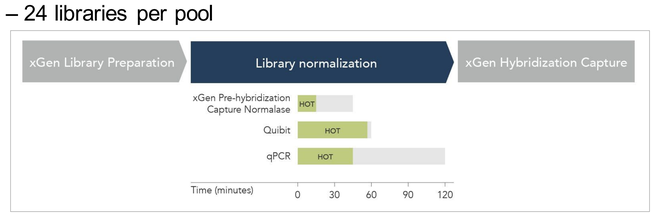

Figure 1. The xGen Pre-Hybridization Capture Normalase Module reduces workflow time. Hands-on-time (HOT) and total workflow (grey bars) are both substantially faster than Qubit or qPCR. Times determined using 24 libraries.

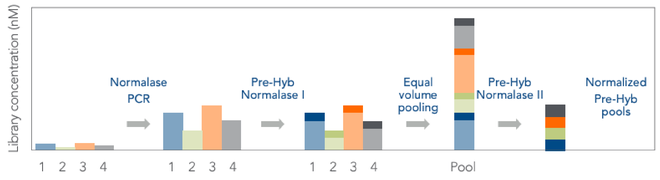

Figure 2. Main steps in the Pre-Hybridization Capture Normalase workflow:

1. During Normalase PCR, Normalase primers are used to generate 3x the minimum library yield needed for downstream hybridization capture.
2. The Pre-Hyb Normalase I step enzymatically selects a user-defined library fraction.
3. Equal volumes of libraries are then pooled creating a multiplex pool for hybridization capture.
4. Pre- Hyb Normalase II step enzymatically generates an equimolar library pool that is cleaned and primed for use in hybridization capture.

xGen Pre-Hybridization Normalase outperforms bead-based normalization

- Proprietary enzymatic normalization using xGen Pre-Hybridization Normalase results in less sequencing read variation (CV = 9.3%) than a commercially available bead-based normalization library prep (CV=11.9%) **(Fig. 3A)**
- The bead-based method produced a read range nearly twice as high as the Normalase solution **(Fig. 3A)**
- By generating libraries with more uniform sequencing reads **(Fig. 3A),** higher diversity **(Fig. 3B)**, higher coverage **(Fig. 3C),** lower duplication rates **(Fig. 3D)**, fewer sequencing reads are being discarded during sequencing and analysis, resulting in cost-savings

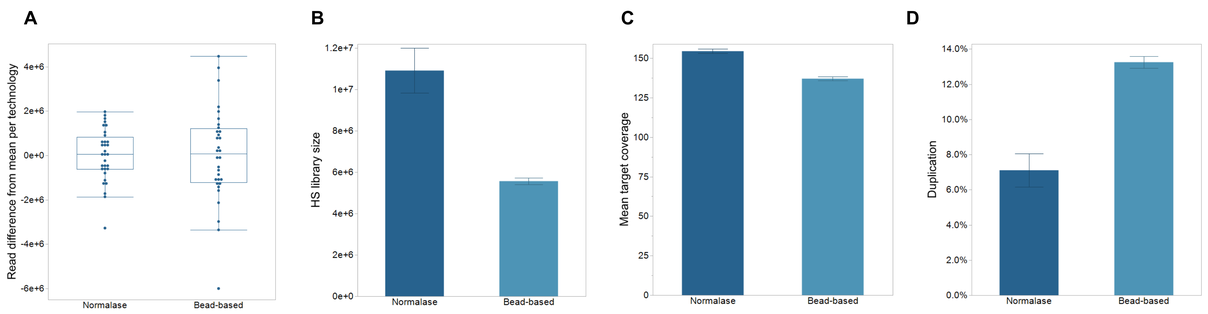

Figure 3. Libraries were generated with 50ng of a Myeloid DNA Reference Standard (Horizon) using xGen cfDNA & FFPE with xGen Normalase UDI Index primers and the xGen Pre-Hybridization Capture Normalase Module (N=32), or a commercially available bead-based library prep (N=32). Hybrid capture was performed as two16-plex captures per library type using xGen AML Cancer Hyb Panel and xGen Core Capture Reagents. All libraries were sequenced on an Illumina NextSeq 2000, subsampled after read count analysis to 4 M/ sample and analyzed using Picard. Error bars represent standard deviations.

xGen Pre-Hybridization Normalase results in lower sequencing read CV than Qubit across a range of sample input amounts

- Library normalization using xGen Pre-Hybridization Normalase results in less sequencing read variation than using Qubit to quantify individual libraries
- Increased throughput achieved with xGen Pre-Hybridization Normalase by streamlining workflows with multiple inputs into library prep and increase efficiency by normalizing multiple library input amounts using the same PCR cycling conditions: 10ng, 20ng, and 50ng **(Fig. 4A),** 5ng, 10ng, and 20ng inputs **(Fig. 4B)**, and 1ng, 5ng, and 10ng inputs **(Fig. 4C)**
- Individual quantification using Qubit takes more time **(Fig. 1A)** and results in more read variation than enzymatic normalization (**Fig. 4**)
- When evaluating the cost of reagents and hands-on-time, workflows using Qubit for quantification and individual library normalization costs ~2.5 more than using xGen Pre-Hybridization Normalase, and qPCR (KAPA) costs ~3 times more

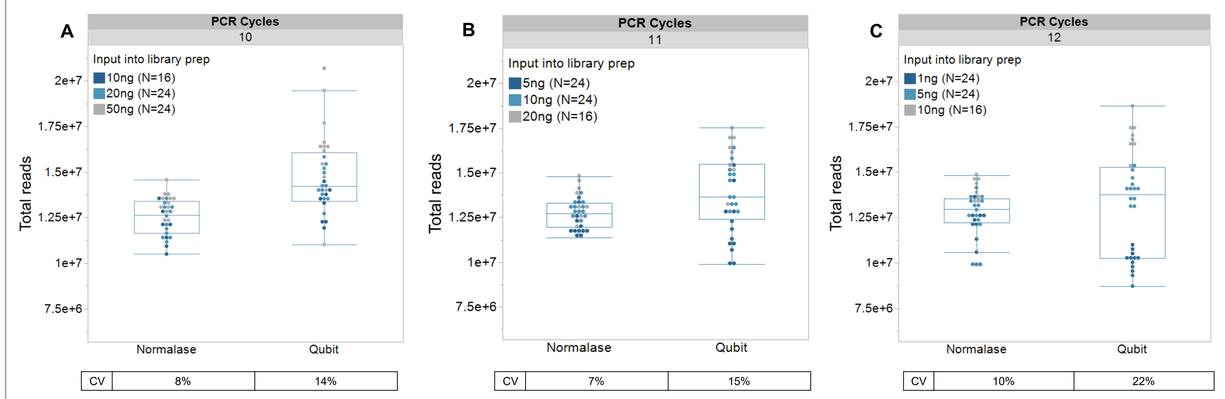

Figure 4. xGen DNA EZ Libraries were generated with xGen Normalase UDI Index primers and xGen Pre-Hybridization Capture Normalase Module with varying PCR cycles and inputs of Coriell gDNA (NA12878). Hybrid capture was performed as eight 24-plexes, each capture included libraries from each PCR plate, using xGen AML Cancer Hyb Panel and xGen Core Capture Reagents. Captured libraries were sequenced on an Illumina NextSeq 2000 and analyzed using Picard.

xGen Pre-Hybridization Normalase maintains expected variant allele frequencies

- Unlike other normalization methods, xGen Pre-Hybridization Capture Normalase Module is not limited to use with only high sample input amounts and can be used according to xGen library preparation input recommendations (down to 1ng)
- Using only a 20 ng DNA input into library prep resulted in low read CV and quality sequencing metrics **(Table 1)**
- Because the read CV was low, no subsampling was performed allowing for all reads to be used during analysis, and resulted in similar target enrichment metrics across all libraries **(Table 1, Fig 5)**
- All 32 replicates identified the 20 expected variants resulting in a 100% sensitivity* down to an AF of 4.8%
- High correlation (R^2 =0.992) between expected and observed allele frequencies (AF) **(Fig. 4B)** showing that the enzymatic normalization process does not negatively impact downstream variant calling

Table 1

Capture replicate	Total reads (CV)	Mean selected bases (%)	Mean target coverage	Mean HS library size
1	6.1%	84.7	961	1.17e + 7
2	8.1%	84.8	922	1.15e + 7

Fig. 5

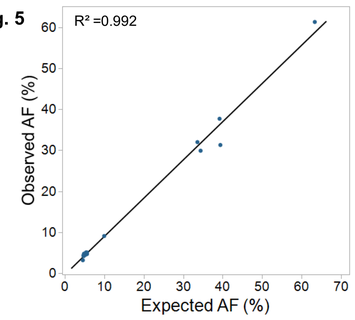

Table 1 and Figure 5. Libraries were generated with an input of 20ng of a Myeloid Reference Standard using the xGen cfDNA & FFPE library kit with xGen Normalase UDI Index primers and the xGen Pre-Hybridization Capture Normalase Module (N=32). Hybrid capture was performed as 2 16-plex captures using xGen AML Cancer Hyb Panel and xGen Core Capture Reagents. Sequenced on Illumina NextSeq 2000, targeting 40M reads/ sample, and analyzed using Picard. The Myeloid DNA Reference Standard (Horizon) contains 20 variants, which range in expected allele frequencies (AF) from 4.8%- 63.3%, in the target space of the xGen AML panel.

*Based on the variant calling method used, two of the expected variants may be missed due to filtering and presences of a multi-allelic variant

Conclusions

xGen Pre-Hybridization Capture Normalase Module:

- Saves hands-on-time and allows for increased throughput **(Fig. 1, 2, 4)**
- Results in consistent read CV ≤10% across multiple library preparation kits **(Fig. 3, 4, 5),** and across multiple inputs into library preparation **(Fig. 4)** leading to more predictable sequencing coverage
- Enables higher quality libraries and more consistent read CV then a bead-based normalization method **(Fig. 3)**
- Allows for lower library input amounts then a commercially available bead-based method **(Table 1)**
- Results in lower read CV then Qubit and 2.5 times cheaper than the reagent and time cost associated with Qubit **(Fig. 4)**
- Maintains expected variant detection frequencies downstream of normalization **(Fig. 5)**