APPLICATION NOTE

Low input PCR-free DNA-seq library prep and normalization.

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Key takeaways

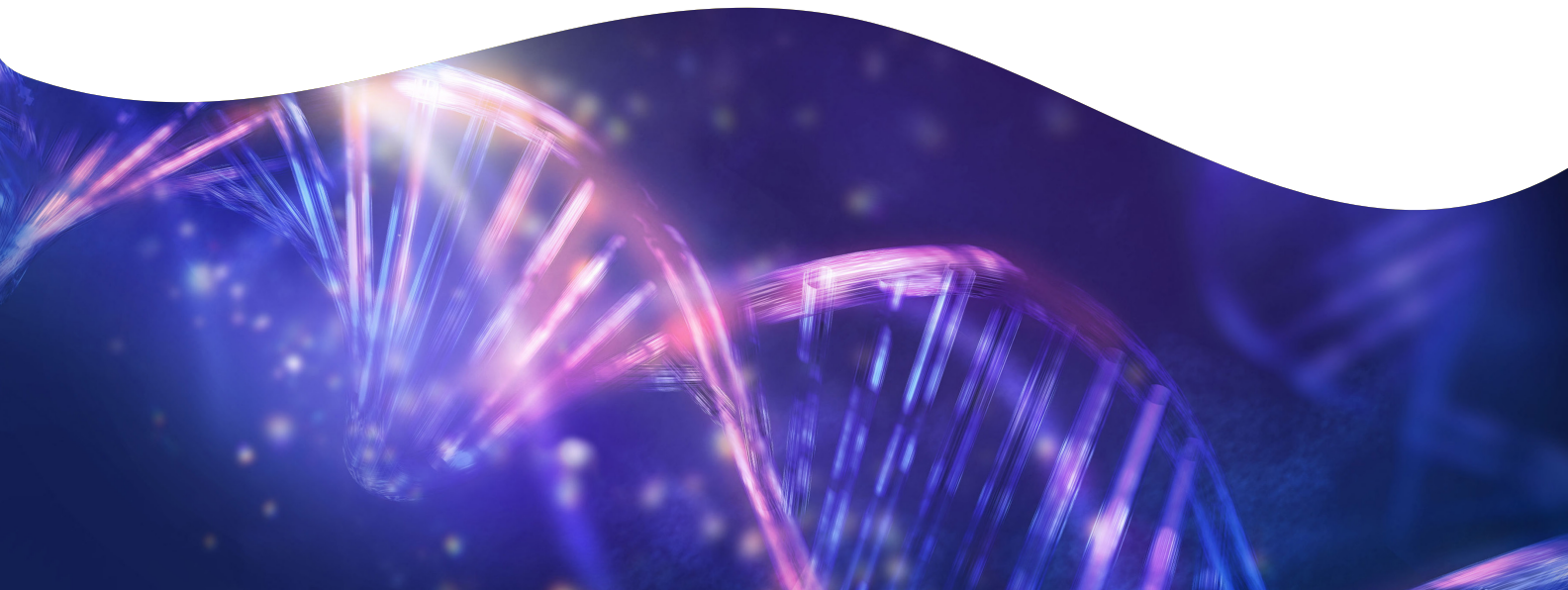
- Eliminate bias introduced during PCR
- Complete workflow enzymatically fragments, prepares, and normalizes libraries

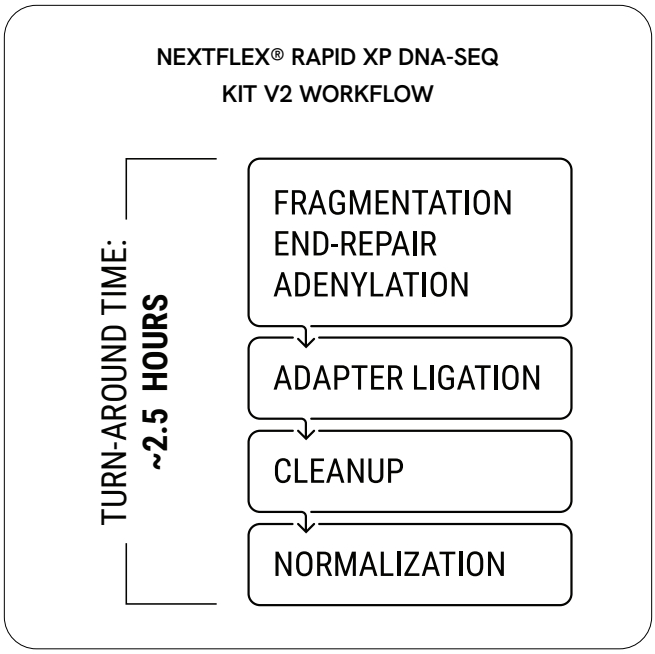
Introduction

During library preparation PCR amplification can cause uneven read coverage, leading to problems in genome assembly and variation analyses. Sequencing genomes with extremely biased composition are particularly affected by this problem[1,2]. To improve data quality, many laboratories performing whole genome sequencing are currently looking for PCR-free workflows.

The NEXTFLEX® Rapid XP DNA-Seq Kit v2 is an enzymatic fragmentation-based DNA sequencing solution which produces consistent results without requiring the traditional normalization steps by using NEXTFLEX® Normalization Beads (included in the kit)[3]. As continuation of this work, we present data supporting the use of NEXTFLEX® Rapid XP DNA-Seq Kit v2 in a PCR-free workflow, in combination with bead-based normalization, using input as low as 100 ng of input. With the incorporation of normalization beads in the protocol, the NEXTFLEX® Rapid XP DNA-Seq Kit v2 has greatly reduced the cost and time required for normalization. Additionally, the protocol is completely automated from library prep to normalization pooling on the Sciclone® G3 NGS/NGSx workstations and Zephyr® G3 NGS workstations.

For research use only. Not for use in diagnostic procedures.





Methods

Purified human gDNA was obtained from Promega (cat no. G1521). Inputs for PCR-free workflow were selected taking into consideration that a minimum amount of DNA is required to fully saturate the normalization beds, assuring consistent normalization. At lower library concentration normalizations can become variable.

Libraries were prepared with NEXTFLEX® Rapid XP DNA-Seq Kit v2 according to the manufacturer's instructions, except that the ligation time was increased from 30 min to 1 hour. Once libraries were prepared, they were pooled equal volume, the pool was quantified with Thermo Fisher® Scientific Qubit® fluorometer and run on an Illumina® MiSeq® platform at 2x75 bp read lengths.

Results

Fragmentation time

Using 100 ng of high quality gDNA as input we tested the impact of different fragmentation times, on insert size.

We observe that 5 minutes is long enough to get inserts with mean size of 275 bp and as expected longer fragmentation times lead to smaller inserts. We therefore recommend using 5 minutes as a starting point for optimization of the fragment size, if using higher inputs or lower quality DNA.

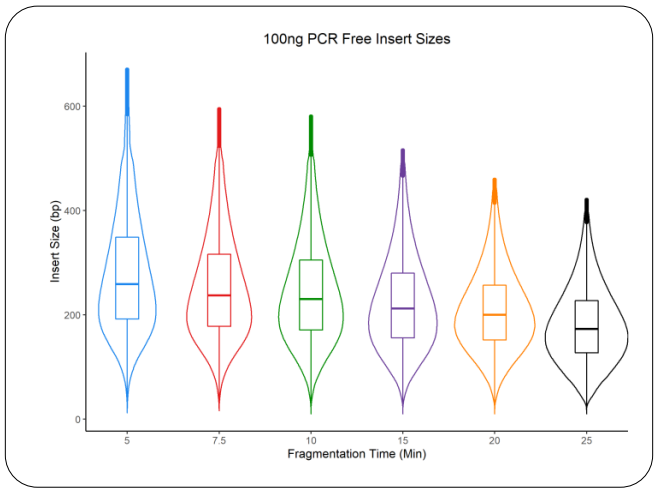


Figure 1: Impact of fragmentation time in the insert size distribution of the libraries obtained

Comparison of read coverage across GC-rich regions

Using 100 ng (Figure 2A) or 400 - 900 ng (Figure 2B) as input we find minimal variability in normalized coverage across 20-60% GC content encompassing ~95% of the human genome (red bars). Coverage is similar regardless fragmentation time and input used to prepare libraries.

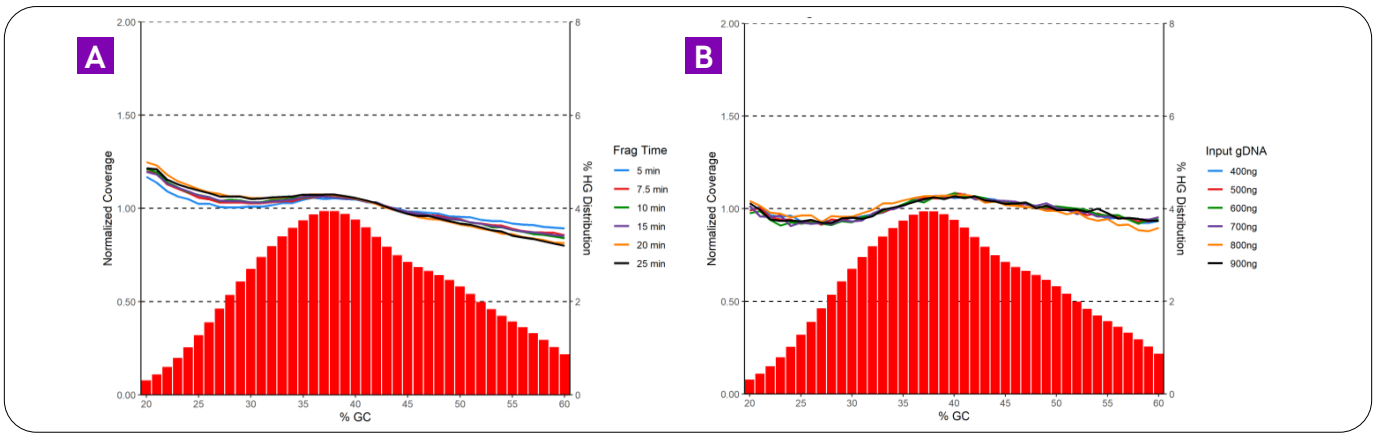


Figure 2: Normalized coverage across 20-60% GC content using 100 ng (A) and 400 - 900 ng of input (B).

Comparison of read coverage across GC-rich regions

The NEXTFLEX® Rapid XP DNA-Seq Kit v2 libraries prepared from a range of DNA inputs demonstrate equivalent passing quality specifications for all DNA inputs.

Input (ng)	Average Q30 score
100	36.27
400	36.17
500	36.22
600	36.13
700	36.10
800	36.13
900	35.99

Yields obtained

We demonstrated that 100 ng of input can be used in the PCR-free workflow. However, to reduce variability of the final library concentrations with bead normalization, we recommend using 200 ng or more. This is consistent with the need to saturate these beads with DNA for highly reproducible normalization across diverse input ranges.

Conclusion

PCR-free workflows are needed because PCR amplification of NGS libraries can cause uneven read coverage, resulting in introducing bias in samples.

With a turn-around time of only 2.5 hours, the NEXTFLEX® Rapid XP DNA-Seq Kit v2 is a convenient workflow that can be used to enzymatically fragment, prepare, and normalize PCR-free libraries from a wide range of starting input. The low variability in normalized coverage contrasting with highly variable input amounts illustrates that benefits the NEXTFLEX® Rapid XP DNA-Seq Kit v2 in constructing PCR-free NGS libraries.

References

1. PCR-free whole exome sequencing: Cost-effective and efficient in detecting rare mutations | PLOS ONE)
2. Performance characterization of PCR-free whole genome sequencing for clinical diagnosis - PMC (nih.gov)
3. Reducing Costs and Time of SARS-CoV-2 Variant Detection by Incorporating Normalization Beads into an NGS Workflow

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