Evaluation of library amplification systems for high stringency applications

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Introduction

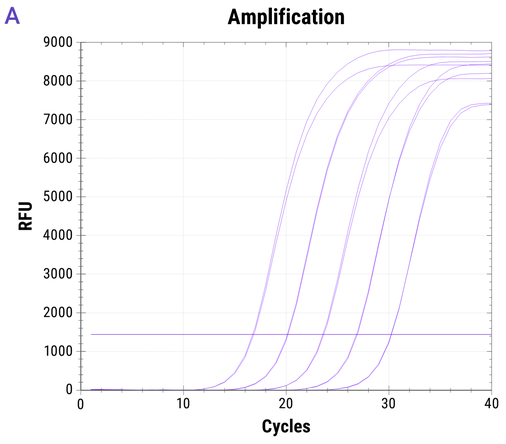
Ideal NGS library amplification achieves faithful representation of the input sample; low-bias amplification enables accurate conclusions. Bias in PCR may assume many forms—an efficient reaction is the foundation for low-bias PCR. Efficient reactions require less PCR cycles, which minimizes accumulation of bias and artifacts. Efficiency must be upheld across a range of GC-content templates, and, even for short-read sequencing, must not bias against longer inserts. In order to support applications that require high yield, e.g., hybrid capture, efficiency must be maintained as product accumulates in the reaction. Efficient reactions generate uniform UMI families, which minimizes sequencing requirements and supports error correction. High-fidelity nucleotide incorporation supports rare variant detection in ctDNA and other applications. Aforementioned performance traits should be maintained in the presence of SPRI and streptavidin purification beads, which are commonly used in library prep workflows. Lastly, a hot start mechanism that efficiently inhibits both polymerase and exonuclease activity enables automated library prep.

High-efficiency Amplification Delivers High Yields

Robust Amplification Across Extreme GC Content

Primer set	Minimum copies tested	Lot	GC content (%)	Efficiency	R^2
P5/P7	3,000	Lot 1	20.8	98.7	0.999
			45.6	98.4	0.999
			80.9	97.5	0.999
		Lot 2	20.8	94.6	0.999
			45.6	100.3	0.997
			80.9	96.6	0.999
		Lot 3	20.8	97.7	1.000
			45.6	97.9	0.999
			80.9	97.4	0.999

Table 1. PCR efficiency and reproducibility at low input across GC extremes



Efficient Amplification when Targeting High Yields

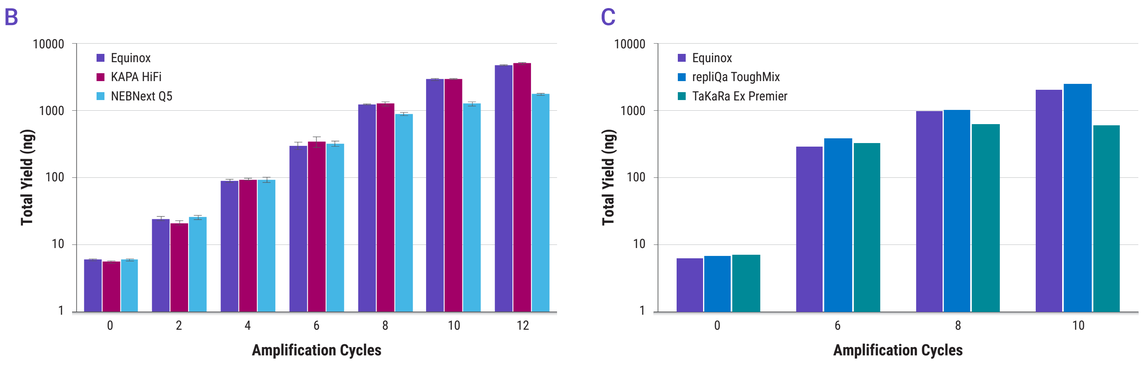


Figure 1. Equinox exhibits efficient amplification and enables high yields for hybrid capture. qPCR with extreme, moderate, and low GC content templates yields excellent efficiency and reproducibility, down to 3000 copies of template (1 femtogram) **(Table 1)** and **(A)**. In two experiments **(B)** and **(C)**, 10 ng of library was used as template for a range of PCR cycle numbers using various master mix providers, then total yield was quantified. As PCR product accumulates, Equinox maintains efficient amplification as well as or better than other mixes. Efficiency drops with NEBNext Q5 and TaKaRa Ex Premier at lower concentrations of PCR product, which is problematic for hybrid capture applications which require high yield.

Uniform UMI Family Coverage

Figure 2. Equinox generates uniform UMI family depths, which increases sequencing economy and enables error correction of more families. (A) Libraries prepared from human genome using UMI-containing adapters were amplified with Equinox, KAPA HiFi, or *Taq*, then sequenced to depths such that each UMI was observed by many raw reads. The ideal line indicates uniform coverage across UMI families modeled with a Poisson distribution. The *Taq*-based system generates non-uniform coverage of UMIs due to preferential amplification of a subset of templates and inefficient amplification of more difficult templates (also known as 'jackpotting'), which results in few bins receiving many reads and many bins not receiving enough reads to enable error correction. **(B)** UMI uniformity and error correction, schematic.

Low-bias Amplification

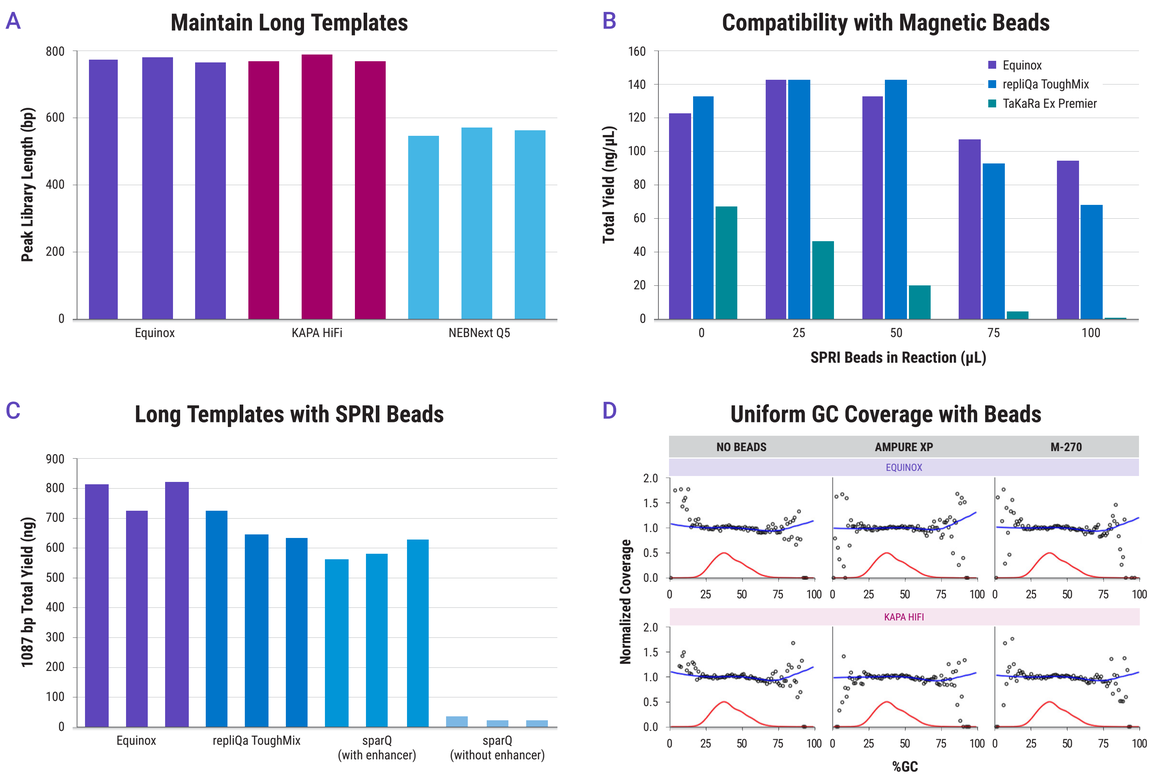


Figure 3. Equinox amplifies long library inserts efficiently, even in the presence of SPRI beads in the reaction. (A) Human genome library for Illumina sequencing with ~600 bp inserts was amplified by 14 cycles of PCR with three amplification mixes. Resulting libraries were assessed for length by D5000 TapeStation. Equinox efficiently amplified the long libraries while Q5 heavily biased shorter templates, which also resulted in significantly reduced yield. **(B)** Similarly, as SPRI beads are titrated into the reaction, yield from TaKaRa Ex Premier drops significantly. **(C)** A 1 kb template flanked with P5/P7 was amplified for 26 cycles in the presence of short templates and yield was quantified. These reactions contained 100 µL of washed SPRI beads to stress the reaction. Equinox generated robust yields in the presence of the beads. sparQ is not compatible with beads without the included enhancer solution. **(D)** Human genome libraries amplified in the presence or absence of purification beads were sequenced and GC content was unaffected.

Ultra-high Fidelity Enables High-sensitivity Applications

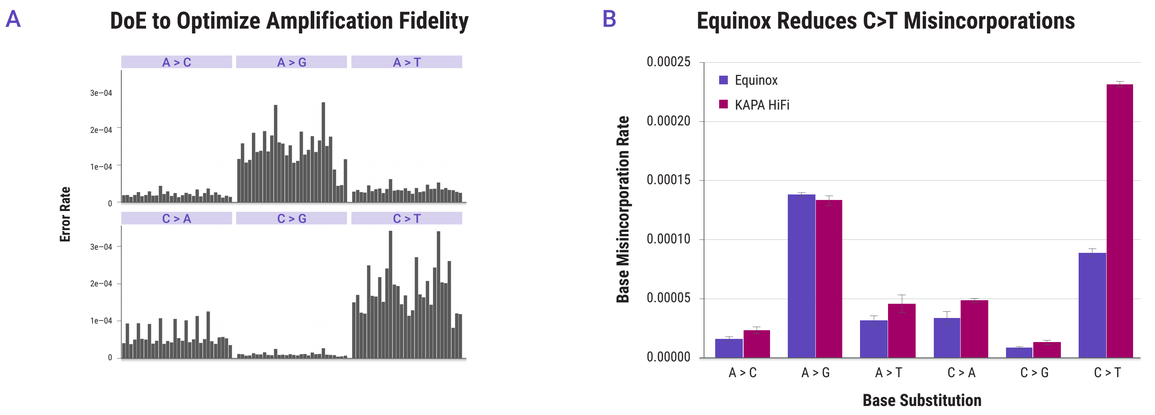


Figure 4. Equinox exhibits industry leading fidelity. (A) A design of experiments approach to optimizing fidelity demonstrates that many characteristics in the reaction, from enzyme variant selection to buffer composition, influence fidelity. **(B)** After optimizing fidelity and many other desired library amplification performance characteristics, the resulting product reduces C>T misincorporations significantly.

Improved Hot Start Antibody Formulation

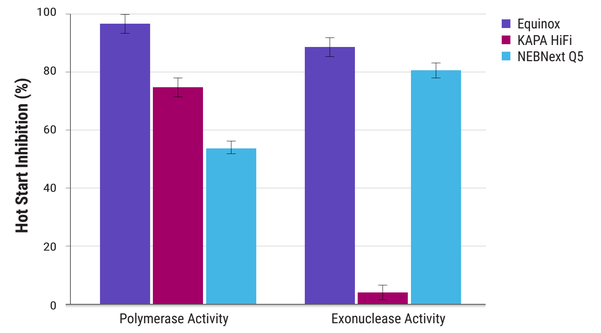


Figure 5. Hot start function enables automated library amplification. Polymerase and exonuclease activities of Equinox polymerase, KAPA HiFi HotStart DNA Polymerase and NEBNext Q5 DNA Polymerase were assessed by the detection of dNTP incorporation or dNMP release, respectively, after incubation at 25°C. Percent inhibition is reported relative to uninhibited formulations.

Conclusions

- Equinox has excellent efficiency across a broad range of GC contents, from 20.8% to 80.9%, does not bias against long library inserts, and tolerates purification beads in the reaction.
- As product accumulates in the reaction, Equinox maintains amplification efficiency as well as or better than other mixes; this is important for hybridization capture workflows
- Equinox generates uniform UMI families which supports economical sequencing and powers error correction
- Equinox exhibits excellent fidelity
- Near complete hot start inhibition of both polymerase and exonuclease activities make Equinox the best choice for automated library amplification