repliQa HiFi ToughMix: Best Practices for Optimal PCR Amplification

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ABSTRACT

DNA polymerases were optimized for diverse PCR and sequencing applications. However newer, more advanced applications have created the need for a next-generation DNA polymerase. repliQa HiFi ToughMix was specially formulated to provide high processivity, high sensitivity and high fidelity for a wide range of targets. In this application note, we show the benefits of repliQa HiFi ToughMix and provide guidelines for optimal PCR amplification.

INTRODUCTION

The Polymerase Chain Reaction (PCR) has contributed to significant advancements in scientific research and healthcare. However, much of this success lies on the evolution of the DNA polymerase, *Thermus aquaticus* (*Taq*), enzyme that mediates the amplification of DNA sequences during PCR cycles. Since its discovery, there have been significant engineered improvements such as the addition of HotStart antibodies and the development of high-fidelity DNA polymerases allowing for faster, more reliable and more accurate PCR amplification.

DNA polymerases have become indispensable for various applications including gene expression analysis, sequencing, cloning, diagnostic testing and others. Nonetheless, major advancements in other applications like next-generation sequencing (NGS), single nucleotide polymorphism (SNP) detection, and whole genome amplification (WGA) have increased demand for novel DNA polymerases with exceptional properties. Desirable attributes are specificity, thermostability, fidelity, processivity, long range capability, inhibitor tolerance and ability to amplify high GC targets. However, most polymerases perform well for only a few of these traits. As a solution, Quantabio developed repliQa HiFi ToughMix which addresses all of these attributes and more.

repliQa HiFi ToughMix is a unique next generation PCR master mix that provides 90 times higher fidelity compared to wild-type *Taq*, while showing extreme speed with extension times as fast as 1–10 sec/kb depending on target length. repliQa HiFi ToughMix is a highly sensitive HotStart polymerase with long range capabilities. It can amplify fragments as long as 24 kb of genomic DNA and with varying GC-content. Additionally, this enzyme is coupled with Quantabio's industry leading ToughMix technology, making it tolerant to a wide range of PCR inhibitors and further improving the versatility of this enzyme. In this application note, we present the unique features of repliQa HiFi ToughMix along with guidelines for optimal PCR results.

METHODS

Sample Input

DNA from *Escherichia coli* strain K12 (ATCC, 10798D-5), *Fusobacterium nucleatum* (ATCC, 25586D-5), and *Bortadella pertussis* (ATCC, BAA-1335D-5) were used as the template for PCR with a final input of 20 ng per reaction. Genomic DNA from *F. nucleatum*, *E. coli* and *B. pertussis* were mixed in 1:1:1 ratio (hereby referred to as Mixed Bacterial Genomic DNA or MBGD) and inputs of 10 ng and 100 ng were used to prepare whole genome libraries using the sparQ DNA Frag & Library Prep Kit.

PCR amplification

1. Primer sets were picked to amplify specific regions from *E. coli*, *F. nucleatum*, and *B. pertussis* genomes. These targets were between 278 bp and 20 kb (Table 1).

Species	Primer	Sequence 5'-3'	T_m °C*	Amplicon size (bp)
E. coli	Fw	TGGGATCATCGCGGCCATT	68	929
	Rv	GACGCCCCACCAGGTTGAGA		
	Fw	GTGTGAAAACTGGGCGCAAG	64	1012
	Rv	ACCACTGCCAATCTGCGAAA		
	Fw	ATGTGATGCGGTGACGTTTA	60	949
	Rv	CATTGTGACGTTGGTTCTGG		
	Fw	CAACGTTCAACTCTGGTAGC	56	1007
	Rv	GGAACTGTCACAAGGAATGA		
	Fw	ATTTTTCGTGGATTCTAATG	52	939
	Rv	AGATGACATTAAGGAAAACG		
	Fw	GGTATTTGACGTGCGTCCTT	60	278
	Rv	TATACGGCGCGGTAATATCC		
	Rv	CCAGTGGCTACCGCTGTATT	60	475
	Rv	CGTTGACTAACCCCGGATAA		779
	Rv	ATTGACCATCCGGTGAGAAG		1085
	Rv	ATGGGTTCAGGCTCATTACG		2682
	Rv	GCCGGTAAAACTGTTCCGTA		4882
	Rv	GCTCAAGGCCTTTCAGTGAC		9365
	Rv	CTCACGCTGCAAATCCACTA		15073
	Rv	GTCATGCTCAACCCGTAGGT		19815
F. nucleatum	Fw	GAACAACAATGAGCGCAGAA	60	422
	Rv	TTGAATTTCTGCTGGTGCTG		
	Rv	TCCCATTGTTTAGCCTCCTTT		1133
	Rv	TGTAGAAATTGCCCCTGCTT		5356
B. pertussis	Fw	CACCACGTGAACGACAATTC	60	480
	Rv	GACCACGCTGGATGGTTATC		
	Rv	CCTATGCGTTCCTGTTCCAG		957
	Rv	GCCGCTGATTTACGCTGTAT		4943

Table 1 **Primer sets for PCR amplification with repliQa HiFi ToughMix.**

* The annealing temperature used was the same as the melting temperature (T_m) calculated using Primer3Plus[2] (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) with default parameters.

2. The repliQa HiFi ToughMix master mix was prepared according to Table 2.

Component	Volume per reaction (µL)
repliQa HiFi ToughMix (2X)	10
10 µM Primer mix	1
Nuclease-free water	9 – x
Template DNA	x
Final volume	20

Table 2 **Reaction setup for PCR amplification using repliQa HiFi ToughMix.**

3. The PCR was run according to the cycling programs detailed in Table 3.

2-Step cycling	Temperature	Incubation time	Cycles
Temperature	98°C	30 sec	1
Denaturation	98°C	10 sec	35
Extend	68°C	1 sec	
Hold	4°C	Hold	1

3-Step cycling	Temperature	Incubation time	Cycles
Temperature	98°C	30 sec	1
Denaturation	98°C	10 sec	35
Anneal	T_m °C*	5 sec	
Extend	68°C	1 – 10 sec/kb**	
Hold	4°C	Hold	1

Table 3 **2-Step and 3-Step cycling programs for PCR amplification using repliQa HiFi ToughMix.** 2-Step cycling was used to amplify with primer pairs with a T_m > 63°C. Otherwise, a 3-Step cycling program was used.

* The annealing temperature used was the same as the T_m calculated using Primer3Plus[2].

** The extension time is based on the fragment size: 1 sec for <1 kb; 5 sec/kb for 1–10 kb; 10 sec/kb for >10 kb.

Library preparation and Sequencing

1. Whole genome libraries were prepared from Mixed Bacterial Genomic DNA (MBGD) using sparQ DNA Frag & Library Prep Kit following the product manual (IFU-122.1-REV-08-95194-sparQ-DNA-Frag-Library-Prep-Kit-0623). MBGD of 10 ng and 100 ng were fragmented for 16 min and 10 min respectively at 32°C to achieve a target fragment size of 350 bp. Libraries were prepared by adding 20 nM or 250 nM sparQ UDI Adapters in the presence of DNA Ligation master mix for 10 ng or 100 ng of input DNA respectively. The

ligation reaction was incubated at 20 °C for 15 min. Libraries were purified using sparQ PureMag Beads and eluted in 10 µL elution buffer (10 mM Tris-HCl, pH 8.0). Adapter ligated libraries were amplified with either sparQ HiFi PCR Master Mix with a standard 30 sec extension time or repliQa HiFi ToughMix with either a 1 sec or 30 sec extension time. PCR amplification was performed at 9 or 4 cycles for 10 and 100 ng inputs accordingly.

2. Libraries were normalized to 4 nM using EB buffer (QIAGEN®). 10 µL of each normalized library with unique indexes were pooled together in a new tube. Pooled libraries were sequenced using MiSeq® Reagent Kit v3 (600 cycles) (# MS-102-3003, Illumina®) using manufacturer's recommendations.

PCR Product and Library Quality Control

1. To verify the size of the PCR products and libraries, 1 µL of the DNA was run on a D5000 ScreenTape on the 4200 TapeStation® System (Agilent).
2. For the long range amplified targets, 1 µL of the DNA was run on a Genomic DNA ScreenTape on the 4200 TapeStation System (Agilent).

RESULTS

repliQa HiFi ToughMix is a fast, high-fidelity mastermix that requires attention when choosing cycling conditions. The annealing temperature, extension time and GC-content are critical parameters to consider when selecting the cycling program and conditions to guarantee a successful PCR.

First, choose between the 2-step or 3-step cycling program by checking the melting temperature (Tm) of the primer sequences using a primer T_m calculator. In this application note, we used the default settings of the Check Primers function on Primer3Plus[2]. Following the manual for repliQa HiFi ToughMix the 2-step PCR cycling program is recommended when the primers' T_m are at least 63°C, otherwise 3-step cycling is preferred.

Primer T_m determines the cycling program

Using *E. coli* DNA as a genomic DNA template, we amplified a ~1 kb target using the 2-step PCR cycling program and primers with melting points ranging from 52°C to 68°C (Figure 1). As the primer T_m increased, the success of target amplification also increased, demonstrating that repliQa HiFi ToughMix has improved results using a 2-step PCR cycling program when working with primer T_m greater than 63 °C.

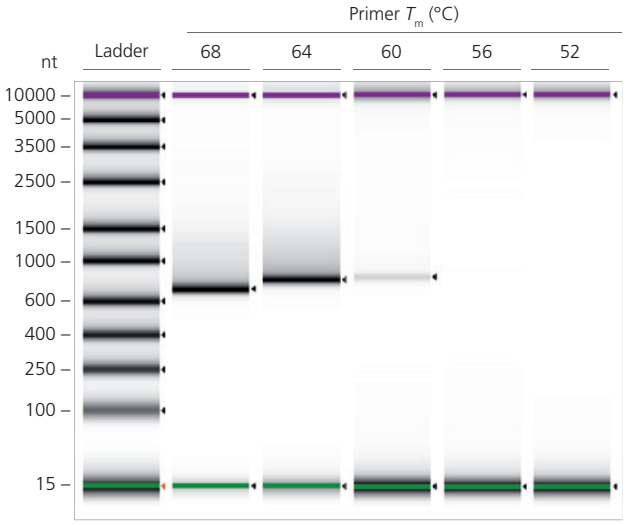

Figure 1 **repliQa HiFi ToughMix in a 2-step PCR cycling program.** TapeStation (D5000 ScreenTape) analysis of ~1 kb targets from *E. coli* DNA using primer sets with different melting temperatures.

Cycling program for primer sets with lower T_m

We used the 3-step cycling program to amplify the same ~1 kb targets from *E. coli* DNA for the primer sets with T_m < 63°C. We adjusted the annealing temperature according to the Tm calculated by Primer3Plus[2] and used 1 second for the extension time. All targets were successfully amplified regardless of the primer T_m (Figure 2). This demonstrates that repliQa HiFi ToughMix requires 3-step cycling to successfully amplify targets with primer T_m < 63°C and still benefit from its high processivity.

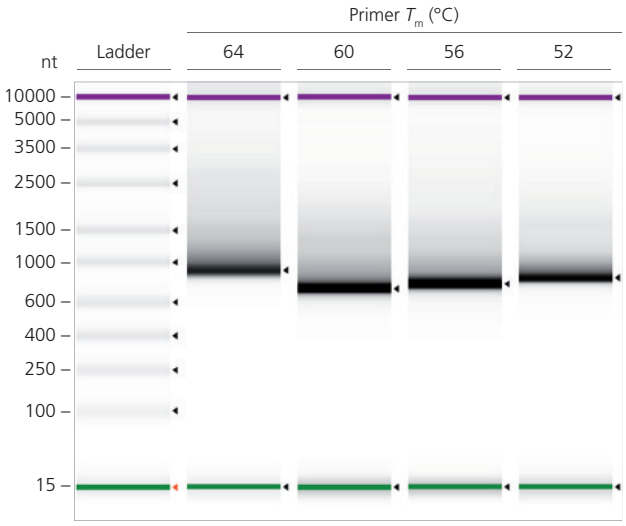

Figure 2 **repliQa HiFi ToughMix in a 3-step PCR cycling program.** TapeStation (D5000 ScreenTape) analysis of ~1 kb targets from *E. coli* DNA using primer sets with different melting temperatures.

Long Range Amplification

repliQa HiFi ToughMix also has long-range amplification capabilities. Targets from 250 bp up to 20 kb were amplified from *E. coli* DNA using repliQa HiFi ToughMix. The extension times of 1 sec for targets < 1 kb, 5 sec/kb for 1–10 kb and 10 sec/kb for >10 kb were used during a 3-step cycling amplification based on the target length. TapeStation electrophoresis confirmed the sizes of the DNA fragments (Figure 3). The results clearly demonstrate that repliQa HiFi ToughMix is a versatile amplification reagent that can be used for both smaller targets and long-range amplification without compromising PCR yield.

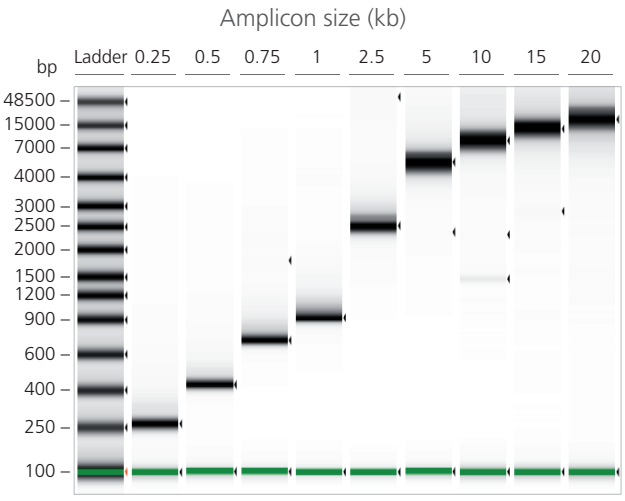

Figure 3 **repliQa HiFi ToughMix long range amplification.** TapeStation (Genomic DNA ScreenTape) analysis of targets with different lengths (278 bp to 20 kb) were amplified using from *E. coli* DNA using repliQa Hifi Toughmix.

Wide GC Tolerance Range

F. nucleatum, *E. coli*, and *B. pertussis* genomic DNA have varying range of GC-content, 27%, 50% and 67% respectively. We used them as templates to amplify three targets with different sizes (500 bp, 1 kb and 5 kb) in a 3-step cycling program using repliQa HiFi ToughMix. This specially formulated master mix was able to successfully amplify all nine targets with varying GC-content and length as visualized by TapeStation electrophoresis (Figure 4). Furthermore in a previous application note, we demonstrated that enhancers like DMSO and betaine can increase repliQa HiFi ToughMix's ability to amplify targets with high GC-content (>70%) without inhibition of the polymerase activity (MK-AN-0015_REV_02_Optimal_PCR_Amplification_0723_lr).

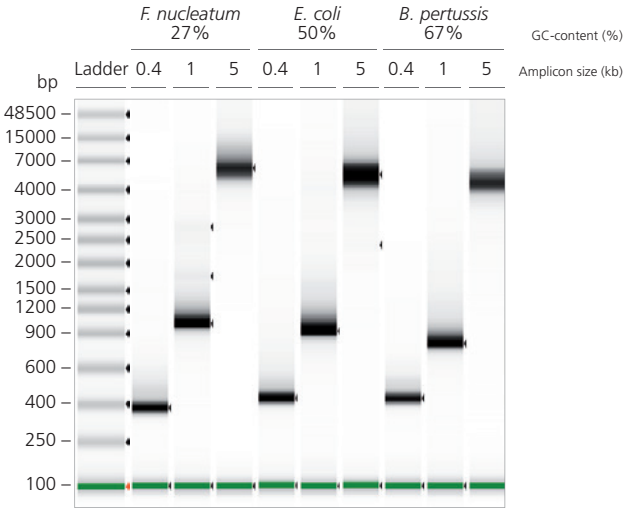

Figure 4 **repliQa HiFi ToughMix is high-GC tolerant.** TapeStation (Genomic DNA ScreenTape) analysis of the amplification of targets of different sizes (0.5 kb, 1 kb, 5 kb) from genomes with varying GC-content: *F. nucleatum* (27% GC), *E. coli* (50%), and *B. pertussis* (67%).

Whole Genome Library Amplification

Inputs of 10 ng and 100 ng of Mixed Bacterial Genomic DNA (MBGD) were used to prepare whole genome libraries using the sparQ DNA Frag & Library Prep Kit. Libraries were amplified with either sparQ HiFi PCR Master Mix with a standard 30 sec extension time or repliQa HiFi ToughMix with either a 1 sec or 30 sec extension time (Figure 5 A). Similar library yields were generated with both mastermixes even at lowest input of 10 ng with 9 PCR cycles of amplification, suggesting that repliQa HiFi ToughMix produced high library yield with only 1 sec of extension time (Figure 5 B). According to the sequencing metrics, both sparQ HiFi PCR Master Mix and repliQa HiFi ToughMix showed low duplication rates regardless of the DNA input amount or the number of PCR cycles (Figure 5 C). Finally, the genome coverage across a wide range of GC content was identical for both mastermixes showing that sparQ HiFi PCR Master Mix and repliQa HiFi ToughMix have little bias towards different GC content (Figure 5 D).

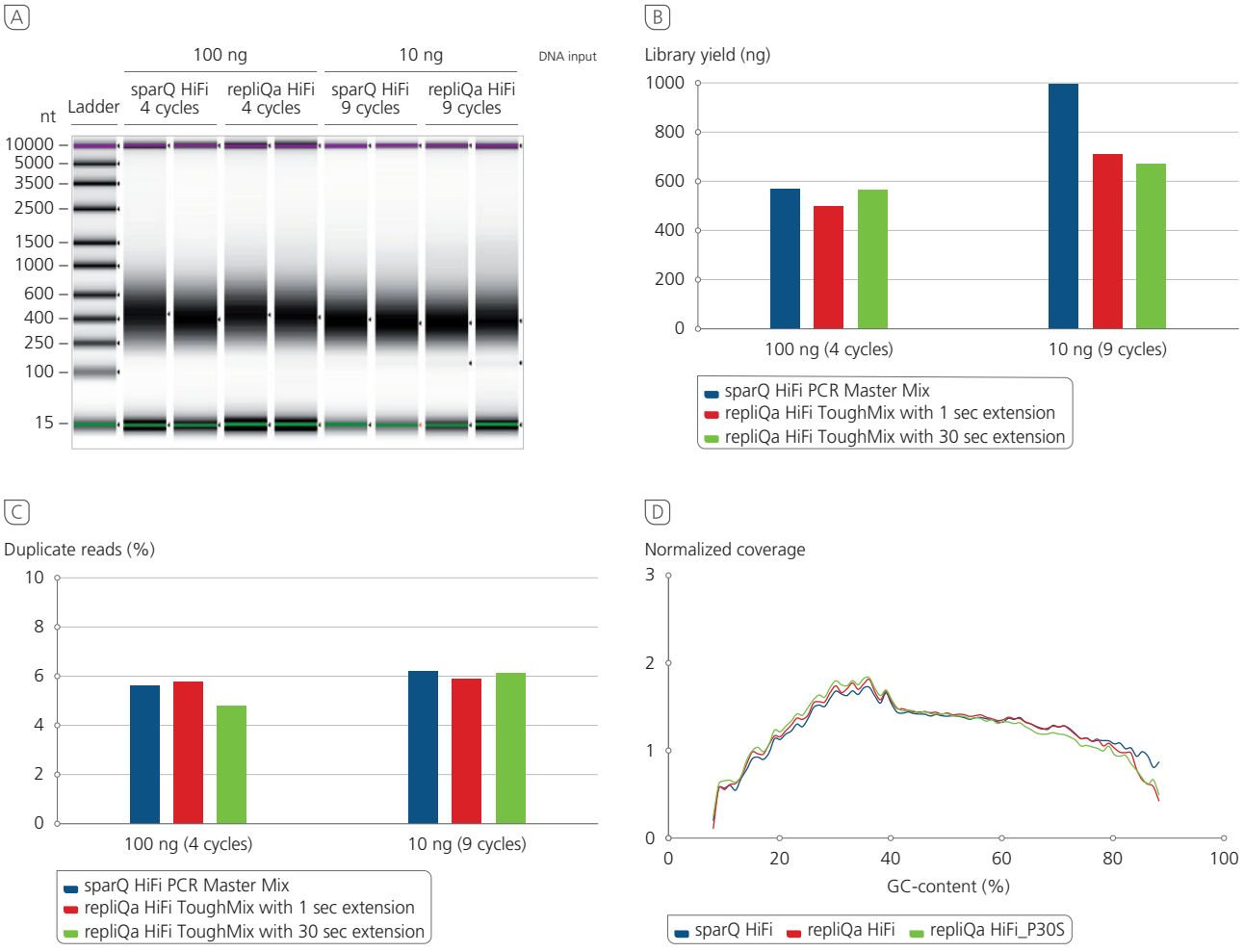

Figure 5 **Whole Genome library amplification with repliQa HiFi ToughMix.** **A** TapeStation (D5000 ScreenTape) analysis of the prepared libraries using sparQ Frag & Library Prep Kit from 10 ng and 100 ng MBGD input with 4 and 9 cycles of PCR amplification using sparQ HiFi PCR Master Mix and repliQa HiFi ToughMix with 1 sec or 30 sec extension time respectively. **B** Library yields, **C** Duplication rates, **D** Genomic coverage across GC-content.

CONCLUSIONS

repliQa HiFi ToughMix stands out over other high-fidelity polymerases due to its remarkable features: inhibitor-tolerance, long range amplification abilities, suitability for wide GC-content ranges, and fast speed. The Quail team at the Wellcome Sanger Institute chose repliQa HiFi ToughMix as the best polymerase for short and long read sequencing over 20 other polymerases.[3] repliQa HiFi ToughMix showed outstanding performance considering parameters like yield, fidelity, polymerase reads, low coverage index, and sub-read lengths. Additionally, repliQa HiFi ToughMix offers other advantages including tolerance to wide range of PCR inhibitors, allowing it to work with crude lysate or dirty samples while eliminating expensive and time-consuming purification steps. Stein *et al*. preferred repliQa HiFi ToughMix for its inhibitor-tolerance and high-speed for development of a high speed 16S DNA barcoding PCR from crude extractions of soft-bodied insects.[4] Finally, repliQa HiFi ToughMix can amplify from templates containing uracils (dU) or using primers containing inosines (dI) and uracils (dU), making it ideal for applications like DNA methylation analysis or bisulfite genomic sequencing. Overall, repliQa HiFi ToughMix has impressive features and can be used for a broad range of applications. Successful PCR amplification can be ensured by following the guidelines described in this application note.

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