Technical Note

Amplification of Long PCR Amplicons Using Philisa*FAST*™ DNA Polymerase

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Introduction

The purpose of this study was to use a new hot start, high fidelity polymerase in combination with a rapid thermal cycler to amplify PCR products 1 kilobase (kb) and longer. Philisa*FAST*™ is a hot start, highly processive DNA polymerase specifically designed to work with the Philisa® Thermal Cycler, which is capable of rapid heating and cooling ramp rates. Primers were designed to target Lambda Phage genomic DNA and create amplicons with the approximate molecular weights of 1, 2.5, 5, 7.5, and 10 kb. PCR utilized a hot start two-step protocol with a combined extension and annealing time of 17, 30, 90, or 240 seconds. With increased cycling time, eventually each long amplicon target was successfully resolved.

Materials and Methods

As Philisa*FAST* DNA polymerase is powered by Takara Bio Inc. SpeedSTAR™, the instructions for use (IFU) protocol for Takara SpeedSTAR was used as an initial protocol guide.[1] Ten ng (approximately 150,000,000 copies) of target DNA was added to each 25 µL reaction along with: 0.25 µM of each primer, Philisa*FAST* Buffer II (containing 2 mM $MgCl_2$ per reaction), 0.25 mM dNTPs, 0.625 units of Philisa*FAST*, and water to make up volume. For the optimized PCR protocol, 25 µl reactions were also prepared in Philisa*FAST* Buffer II with 2.5 units of Philisa*FAST*, 0.25 mM of both primers, 0.25 mM of dNTPs, 2 pg of Lambda Phage DNA (approximately 50,000 copies) as target, and water to make up volume. All PCR reagents were part of the Streck Philisa*FAST* DNA Polymerase kit (Catalog No.: 250024) except water which was PCR grade from QIAGEN®.

All amplicons were designed using the same forward primer. For each increase in molecular weight, specific reverse primers were designed using NCBI Primer-BLAST[2] and ordered from IDT. These primers are detailed in the table below:

Primer Name	Sequence (5' to 3')	Product Length (BP)
Universal Forward	GGACGGCATTCTGCTCGAT	N/A
Reverse A	CTGCGGCTGGTAATGGGTAA	1000
Reverse B	TTCTTTCGTCCCCGTCAGG	2500
Reverse C	TTCCGGTTGCTGTCTGACTC	5062
Reverse D	CCCGGCTTTTCGCATCA	7500
Reverse E	GCGTCGCTGCCATTTGT	10,000

The thermal cycling protocols for both the initial and the optimized runs are detailed below:

Initial Protocol

Step	Temperature (°C)	Time (seconds)
1) Hot Start	94	60
2) Denaturation	98	5
3) Extension/Anneal	68	300

Repeat steps 2 and 3 for 30 cycles

Optimized Protocol

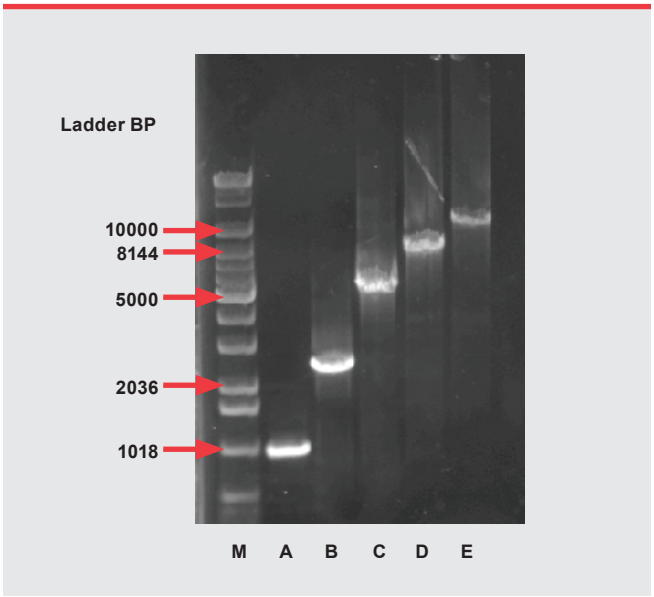
Step	Temperature (°C)	Time (seconds)
1) Hot Start	94	120
2) Denaturation	98	10
3) Extension/Anneal	65	X (see text)
4) Final Hold	65	X (see text)

Repeat steps 2 and 3 for 30 cycles

For the optimized protocol, the hold time (noted as X) was varied with the goal of eventually resolving all targets and time was added in a stepwise fashion to allow detection of the longer amplicons. For both the extension/anneal step and the final hold step, X corresponds to 15, 60, 90, or 240 seconds. When cycled, these protocols equated to total PCR times of 17, 24, 56, and 134 minutes, respectively and each target was resolved within one or more of the listed PCR time-frames.

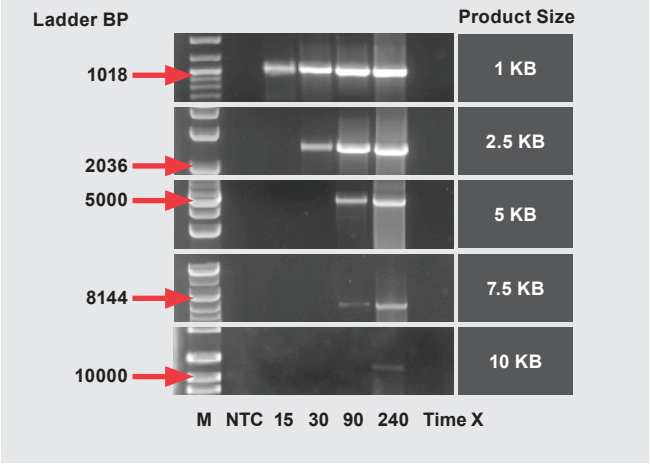
PCR products were loaded and resolved on a 0.7% agarose gel with an appropriate DNA ladder (1 KB DNA Extension Ladder, Invitrogen™, (Catalog No.: 10511-012), stained with ethidium bromide, and imaged with a Bio-Rad® Molecular Imager VersaDoc™ 5000MP.

Figure 1



IFU Based Protocol Gel - the gel results of the initial IFU guided protocol for the Lambda Phage DNA long amplicon PCR. Well M is a molecular weight marker. Wells A, B, C, D, and E are 1, 2.5, 5, 7.5, and 10 kb amplicons, respectively. For gel analysis, 6 uL of PCR product was loaded on a 0.7% gel and run at 70 V for 100 minutes. After electrophoreses, gel was stained with ethidium bromide and imaged on a molecular imager.

Figure 2



Optimized Protocol Gel – the gel results of the optimized protocol for the Lambda Phage DNA long amplicon PCR. The anneal/extension time is noted below the figure in seconds, with NTC (15-second protocol was used for NTCs). For gel analysis, 6 µL of PCR product was loaded on a 0.7% gel and run at 70 V for 80-180 minutes depending on amplicon size. After electrophoreses, gels were stained with ethidium bromide and imaged on a digital imager.

Results and Discussion

When the polymerase manufacturer's IFU were followed for PCR master mix preparation, required target concentration, and thermal cycling protocols, all of the designed amplicons were detected on the gel (Figure 1) with a PCR time of 156 minutes. While this demonstrates PCR amplification of 1 kb and larger targets, results were suboptimal for two reasons: first, data using the manufacturer's recommended protocol demonstrated streaking and smearing within gel lanes, some poorly resolved bands, and what may be some non-specific PCR products. Secondly, the effective starting target concentration was approximately 150 million copies per reaction. Due to these limitations, experiments were done to determine the optimal conditions for this assay.

Based on prior studies, a thermal cycler with fast ramp rates can be used to amplify long amplicon PCR fragments.[3] We also know that modification of PCR cycling times and careful selection of PCR master mix compositions will help optimize rapid PCR.[4] Once all optimization experiments were complete, we determined the target concentration required for product resolution could be reduced from ≈ 150 million copies/reaction to 50,000 copies. Furthermore, by including a higher polymerase concentration and altering cycling protocol temperatures and hold times, the total PCR time was as low as 17 minutes for a 1 kb fragment and 134 minutes for a 10 kb fragment (Figure 2). Optimized extension for the 1 kb fragment required 15 seconds, suggesting a rate of 66 bases per second. This number approaches the maximum theoretical rate as given in the Takara IFU. The 10 kb amplicon, which was the largest in the study, required a 240 second extension time, which equates to 42 bases per second.

Conclusions

The *a priori* advantage of a thermal cycler with rapid ramp rates (up to 15 °C) may not seem like a natural fit for long-amplicon PCR because long amplicon PCR generally requires long hold times to allow the polymerase to replicate thousands or tens of thousands of nucleobases. However, protocol optimization to decrease assay time is possible using an appropriate PCR master mix and polymerase as well as a thermal cycler capable of ramping reaction temperature at extremely fast rates. For an end user beginning a long-amplicon PCR study with Philisa*FAST,* we recommend following the IFU for an initial study but the data described here can serve as a guide for reducing cycling protocol time based on amplicon size. As shown above, further optimization of the polymerase manufacturer's instructions increased sensitivity and specificity, as well as decreased overall reaction time, leading to faster detection of long amplicon products.

Citations

1. Takara SpeedSTAR HS IFU. Catalog No.: RR070A/B Version: 1106Da_b
2. Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen S., Madden, T. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:134.
3. TerMaat, J. Rapid PCR of Long Amplicons Using the Streck Philisa® Thermal Cycler. Streck, Inc., Omaha NE.
4. Zhang, Z., Kermekchiev, M.B., Barnes, W.M. Direct DNA amplification from crude clinical samples using a PCR enhancer cocktail and novel mutants of Taq. *The Journal of Molecular Diagnostics: JMD* 2010, 12(2):152-161.