KAPABIOSYSTEMS

Genetic tests involving long amplicons often require careful optimization, and true high-fidelity amplification for downstream sequencing analyses is not always achievable.

Kapa Biosystems offers two solutions for the routine amplification of long DNA fragments. KAPA LongRange HotStart offers robust amplification in single and multiplex assays, whereas KAPA HiFi HotStart is capable of amplifying human genomic targets >10 kb with an error rate up to 50 times lower than long-range enzyme blends.

Introduction

Some genetic and pharmacogenetic tests require the routine amplification of long DNA fragments from complex genomic targets. Assays targeting fragments >3 kb often require careful optimization of reaction parameters, such as Mg^{2+}, primer and template concentrations and cycling parameters. Gene duplications and pseudogenes represent additional challenges to primer design and reaction efficiency. In cases where downstream sequencing of DNA fragments is necessary to confirm known or identify new alleles and genotypes, high-fidelity amplification is essential.

"Long-range" enzyme blends, consisting of *Taq* DNA polymerase and a small amount of a B-family ("proofreading" or high-fidelity) DNA polymerase, are routinely used for the amplification of long DNA fragments. With these blends, the inherent limitation of *Taq* to extend past 3'-mismatches is overcome through intermolecular transfer of mismatched primer-template complexes from *Taq* to the proofreading enzyme. The 3'→5' exonuclease activity of the latter is capable of repairing the mismatch, thereby restoring the incomplete DNA strand as a template for *Taq* in subsequent cycles of the reaction [1]. The presence of the proofreader results in a slightly (2 – 4 times) lower error rate than *Taq* alone, but does not offer the high-fidelity amplification of pure B-family polymerases (20 – 50 times higher than *Taq)*. Nevertheless, genetic testing involving long fragments typically relies on enzyme blends, as most high-fidelity DNA polymerases are not capable of robust amplification of fragments >3 kb from complex genomic targets.

KAPA LongRange HotStart, a *Taq*-based enzyme blend from Kapa Biosystems, is ideally suited for robust and reliable amplification of long amplicons in single or multiplex assays. The novel, engineered KAPA HiFi HotStart is preferred for tests involving downstream sequencing analyses, as it is capable of amplifying fragments >10 kb from complex genomic targets, with higher fidelity than any other commercially available proofreading enzyme [2]. In this study, pharmacogenetic testing of the cytochrome P450 2D6 gene (see **Figure 1** and inset on p. 2) was selected as a model for demonstrating the success of these enzyme systems in routine long-range PCR.

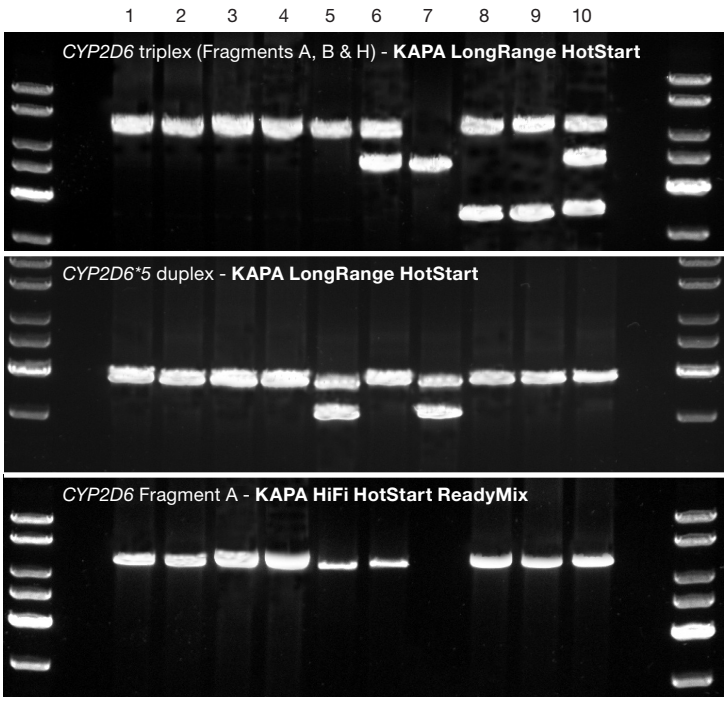

Figure 1. Results of *CYP2D6* genotyping assays.

Results of three *CYP2D6* genotyping assays: the *CYP2D6* triplex (top panel; Fragment A = 6.6 kb; Fragment B = 3.5 kb and Fragment H = 5 kb) and the *CYP2D6*5* duplex (middle panel; 2.9 kb and 3.8 kb internal control) were performed with KAPA LongRange HotStart, whereas high-fidelity amplification of the 6.6 kb Fragment A was performed with KAPA HiFi HotStart ReadyMix (bottom panel). Reactions (25 µl) were set up as described in *Methods and Results*. Cycling was performed simultaneously, using the Biometra T3000 thermocycler and the cycling parameters for the respective enzyme systems listed in **Table 1**. Reaction products (10 µl of KAPA Long Range HotStart and 20 µl of KAPA HiFi HotStart PCR products, respectively) were analyzed in a 0.75% TBE-agarose gel.

Samples 1 – 4 are homozygous for the "normal" *CYP2D6* gene. Genotypes of the other samples are as follows: sample 5 carries both the "normal" and *5 deletion allele, while sample 7 carries a hybrid gene and the deletion. Samples 6 and 7 are both positive for a hybrid gene. Samples 8 and 9 carry gene duplications. Sample 10 is positive for a "normal" *CYP2D6* allele, a hybrid gene and a gene duplication.

Methods and Results

EDTA-blood was obtained from 10 volunteers and genomic DNA isolated using the QIAGEN QIAamp DNA Blood Kit. DNA samples were quantified using the NanoDrop™ 1000 spectrophotometer, and diluted to 10 ng/µl in 10 mM Tris-HCl, pH 8.5.

CYP2D6 triplex and duplex PCRs were performed in 25 µl reaction volumes using the KAPA LongRange HotStart PCR Kit. Reactions contained 1X KAPA LongRange HotStart Buffer, 1.75 mM $MgCl_2$, 0.3 mM of each dNTP, 0.625 U KAPA LongRange HotStart DNA Polymerase, 10 ng human genomic DNA and 1.25 µl of optimized 20X primer premix (**Table 2**). Triplex reactions were supplemented with 5% DMSO. High-fidelity amplification of the full-length *CYP2D6* gene was performed in 25 µl reaction volumes, with 1X KAPA HiFi HotStart ReadyMix, 0.3 µM of each primer and 50 ng of human genomic DNA. Cycling was performed with the Biometra T3000 thermocycler and the cycling parameters for the respective enzyme systems outlined in **Table 1**.

Satisfactory results were achieved for all ten samples with both enzyme systems. In the triplex and *5 duplex assays, even amplification of the different fragments was achieved, allowing for unambiguous genotyping of all individuals. A yield of the 6.6 kb Fragment A sufficient for downstream sequencing analysis was obtained with all samples, except for the deletion mutant (sample 7), for which a full-length fragment was not expected.

Table 1. Cycling parameters used in this study.

Cycling step	Time & temperature	
	LongRange HotStart	HiFi HotStart
Initial denaturation	3 min at 95 °C	3 min at 95 °C
Denaturation	15 sec at 95 °C	20 sec at 98 °C
Annealing/extension	1 min/kb at 68 °C	30 sec/kb at 74 °C
Number of cycles	35	35

Conclusion

KAPA LongRange HotStart and KAPA HiFi HotStart are ideally suited for routine long-range PCR. KAPA LongRange HotStart offers the robustness required for genotyping based on the presence or absence of fragments, particularly in multiplex assays. KAPA HiFi HotStart offers the true high-fidelity and amplification efficiency required to produce sufficient quantities of long fragments for downstream sequencing analyses.

References

1. Cheng, S., *et al.* (1994). *Proc. Natl. Acad. Sci. USA* **91:** 5695 – 5699.
2. KAPA HiFi Cancer Resequencing Case Study (2009). Kapa Biosystems.
3. Gaedigk, A. *et al.* (2007). *Clin. Pharmacol. Ther.* doi: 10.1038/sj.clpt.6100406.
4. Gaedigk, A. *et al.* (2007). *Clin. Pharmacol. Ther.* **81:** 242 – 251.
5. Gaedigk, A. and Coetsee C. (2008). *Eur. J. Clin. Pharmacol.* **64:** 465 – 475.

About the experimental model [3, 4, 5]:

- Cytochrome P450 2D6 is involved in the metabolism of many clinically used drugs.
- Accurate genotyping of the highly polymorphic *CYP2D6* gene is critical to correctly predict metabolizer status prior to clinical trials.
- To date, 78 allelic variants of *CYP2D6* have been defined by the cytochrome P450 nomenclature committee (please refer to http://www.cypalleles/ki.se for full details).
- *CYP2D6* genotype is correlated with ultrarapid (UM), extensive (EM), intermediate (IM) and poor (PM) metabolizer status, with UM and PM types being most at risk for treatment failure as a result of underdosing, or toxicity due to overdosing.
- Genotyping of the *CYP2D6* gene is performed with a panel of long-range PCRs, most importantly a **triplex PCR**, which forms one to three products depending on genotype:
  - **Fragment A (6.6 kb)** encompasses the entire *CYP2D6* gene (but excludes the non-functional *CYP2D7* and *CYP2D8* genes). This fragment serves as genotyping or sequencing template.
  - **Fragment B (3.5 kb)** is amplified if a common gene duplication is present.
  - **Fragment H (5 kb)** is amplified in subjects carrying a *CYP2D7/2D6* hybrid gene.
- Gene deletions *(CYP2D6*5)* are identified with a duplex PCR which targets the *5 deletion fragment (2.9 kb) and an internal control (3.8 kb).
- In the event that new alleles are discovered, the full-length *CYP2D6* gene (6.6 kb Fragment A) is amplified with a high-fidelity polymerase to confirm the genotype by cloning and sequencing the PCR product, or by direct sequence analysis of the PCR product.

Table 2. Primer premixes used in this study.

Name	Primer sequence (5'- 3')	Conc in PCR
Fragment A		
FragA-F	ATGGCAGCTGCCATACAATCCACCTG	0.3 µM
FragA-R	CGACTGAGCCCTGGGAGGTAGGTAG	0.3 µM
Triplex		
FragA-F	ATGGCAGCTGCCATACAATCCACCTG	0.5 µM
FragA-R	CGACTGAGCCCTGGGAGGTAGGTAG	0.75 µM
FragB-F	CCATGGAAGCCCAGGACTGAGC	0.375 µM
FragB-R	CGGCAGTGGTCAGCTAATGAC	0.375 µM
FragH-F	TCCGACCAGGCCTTTCTACCAC	0.5 µM
***5 duplex**		
*5-F	CTCCAGCCTCCACCAGTCCAG	0.5 µM
*5-R	CAGGCATGAGCTAAGGCACCCAGAC	0.5 µM
IC-F	GCATGCACAGCTCAGCACTGC	0.375 µM
IC-R	GCCACCCTGATGTCTCAGTTTCG	0.375 µM

For more information on KAPA LongRange HotStart, KAPA HiFi HotStart ReadyMix or PCR Kits or other Kapa Biosystems products, please contact:

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