Routine GC-rich PCR is a challenging application that requires extensive optimization and often results in low yields, inconsistent amplification, or complete reaction failure.

The novel KAPA2G Robust DNA Polymerase and its proprietary buffer system are ideally suited for the routine amplification of DNA with a GC content >65%. The unique properties of this enzyme include high processivity and tolerance to DNA melting agents at concentrations that inhibit wild-type DNA polymerases.

Introduction

The amplification of GC-rich DNA is often problematic due to stable secondary structures in the template that are resistant to melting. These secondary structures cause DNA polymerases to stall, resulting in incomplete amplification products. Furthermore, GC-rich regions often contain secondary primer annealing sites from which non-specific fragments are amplified. GC-rich PCR typically results in low yields of the target fragment, ladders of non-specific fragments, amplicons of the incorrect length, primer-dimers and/or complete reaction failure. Several methods and additives have been developed to facilitate template denaturation and reaction specificity. Nevertheless, routine amplification of GC-rich amplicons with wild-type DNA polymerases remains unreliable.

Consistent and reproducible amplification of difficult targets is achievable with the highly processive KAPA2G Robust DNA Polymerase, a second-generation enzyme engineered through a process of molecular evolution. The enzyme is supplied with a proprietary reaction buffer designed specifically for GC-rich PCR. In addition to higher success rates, KAPA2G Robust DNA Polymerase offers routine GC-rich PCR in total reaction times up to 50% shorter than those required for wild-type DNA polymerases.

KAPA2G Robust is also available in an antibody-mediated HotStart formulation, which may yield improved results with some primer-template combinations, particularly those that are prone to the formation of non-specific amplification products.

Results

To demonstrate the success of KAPA2G Robust in GC-rich PCR, sixteen amplicons with a GC content ranging from 65% – 85% were amplified from human genomic DNA. Fragments were 240 – 900 bp in length. One of the sixteen amplicons (lane 16 in Figure 1) contains a CGG trinucleotide repeat associated with Fragile X disease. Also included in the experiment was a fragment with an average GC content (50%), amplified with GC-rich primers (lane 1). Results are summarized in Figure 1 and Table 1.

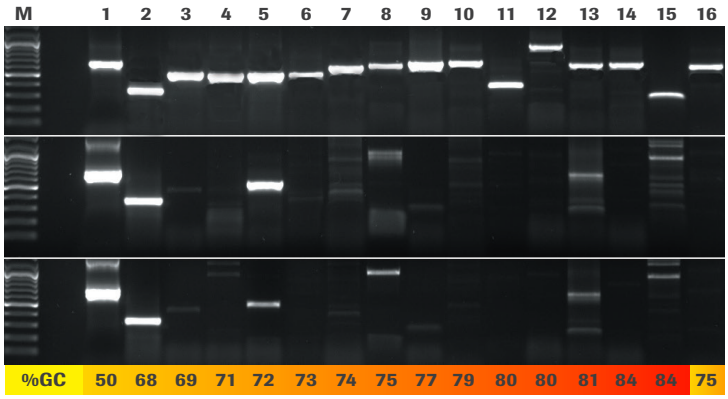

Figure 1. Amplification of GC-rich DNA fragments. Amplification of different types of GC-rich DNA fragments using KAPA2G Robust HotStart (top panel) and two competitor hot start wild-type *Taq* polymerases (middle and bottom panels). Amplicon GC content increases from left (yellow) to right (red), with the exception of the amplicon in lane 16, which has an overall GC content of 75% but contains a CGG trinucleotide repeat. KAPA2G Robust HotStart reactions were performed as described on the next page. Amplicons 1 – 12 (50% – 80% GC) were amplified in KAPA2G GC Buffer, amplicons 13 and 14 in KAPA2G GC Buffer +4% DMSO and amplicons 15 and 16 in KAPA2G Buffer A +1X KAPA Enhancer 1 +5% DMSO. Competitor reactions were performed according to manufacturers' instructions and contained DMSO at a final concentration of 5%. Fragments generated with competitor enzymes for amplicons 7, 8, and 15 were of the incorrect size which do not correspond to the target sequence. All reactions contained 25 ng human genomic DNA as template. Cycling was performed using an Eppendorf Mastercycler epgradient S. Lanes marked M contain DNA ladder.

Table 1. Routine GC-rich PCR success rates and cycling times of KAPA2G Robust DNA Polymerase and competitor enzymes

Enzyme system	Success rate	Cycling time
KAPA2G Robust	100%	36 min
Supplier A	25%	1 hr 10 min
Supplier I	25%	1 hr 03 min

Reaction conditions and cycling parameters

The reaction conditions and cycling parameters given in Tables 2 and 3 are recommended as a starting point for GC-rich PCR using KAPA2G Robust DNA Polymerase. The GC Buffer should always be used as the first approach for amplicons with a GC content between 65% and 80%. For amplicons with a very high (>80%) GC content, or that are recalcitrant to amplification, the GC Buffer +4% DMSO or KAPA2G Buffer A +5% DMSO + 1X KAPA Enhancer 1 should be tried. KAPA Enhancer 1 is a proprietary DNA destabilizer that has similar effects in PCR as betaine. As illustrated in Figure 2, these buffer/additive combinations are not only able to resolve amplicons with a high GC content, but may also be used to improve the yield of target fragments or specificity of amplification.

Table 2. KAPA2G Robust reaction setup for routine GC-rich PCR

Reaction component	Final conc.	Per 25 µL reaction
PCR grade water	–	Up to 25.0 µL
5X GC Buffer or 5X Buffer A	1X	5.0 µL
5X KAPA Enhancer 1 (optional)	1X	5.0 µL
100% DMSO (optional)	4% or 5%	1.0 or 1.25 µL
10 mM dNTP Mix	0.2 mM each dNTP	0.5 µL
Forward primer (10 µM)	0.5 µM	1.25 µL
Reverse primer (10 µM)	0.5 µM	1.25 µL
Template DNA	1 – 50 ng	–
KAPA2G Robust DNA Polymerase (5 U/µL)	1.0 U/reaction	0.2 µL

Table 3. KAPA2G Robust cycling parameters for routine GC-rich PCR

Cycling step	Temperature and time	
Initial denaturation	3 min at 95°C	1
Denaturation	15 sec at 95°C	x35 cycles
Annealing	15 sec at 60°C	
Extension	15 sec at 72°C	
Final extension	0 – 10 min	1

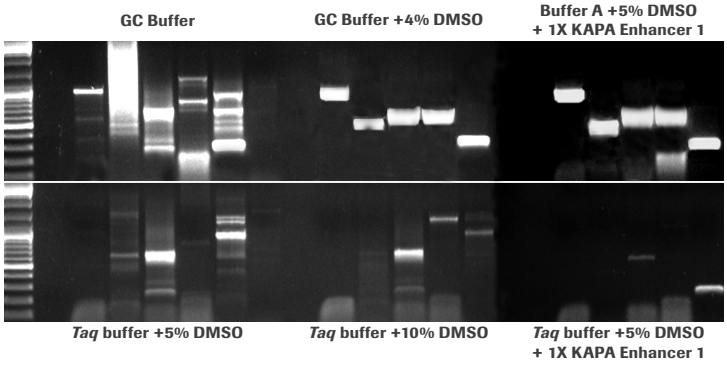

Figure 2. Alternative buffer/additive combinations for GC-rich PCR. Amplification of five GC-rich amplicons (240 – 900 bp) with a GC content >80% with KAPA2G Robust HotStart, using three different buffer/additive combinations (top panel). The GC Buffer yielded unsatisfactory results with all five amplicons. Supplementing the GC Buffer with 4% DMSO, or using KAPA2G Buffer A + 5% DMSO + 1X KAPA Enhancer 1 improved yields and specificity in all cases. Results in the bottom panel were generated with a hot start formulation of wild-type Taq in a standard Taq buffer, supplemented either with 5% DMSO (left), 10% DMSO (middle) or 5% DMSO + 1X KAPA Enhancer 1 (right). The latter resulted in successful amplification of one of the five amplicons. These results clearly illustrate the benefit of using an engineered enzyme that can tolerate melting agents at concentrations inhibitory to wild-type *Taq*. All reactions contained 1U enzyme and 25 ng human genomic DNA as template. Cycling parameters for KAPA2G Robust HotStart reactions were as indicated in Table 2, whereas wild-type *Taq* reactions were performed according to manufacturer's instructions. Cycling was performed using an Eppendorf Mastercycler epgradient S. Lanes marked M contain DNA ladder.

Tips for successful routine GC-rich PCR

- Primer quality is important for successful GC-rich PCR. Always dilute and store primers in TE or 10 mM Tris-HCl, pH 8.0 – 8.5 and not in water.
- To improve yields, the extension time may be increased to 30 sec per cycle. Never anneal for longer than 15 sec per cycle.
- It may be necessary to increase the annealing temperature for specific primer-template combinations to obtain the optimal yield of specific product.
- It is important to fully denature GC-rich targets. Although KAPA2G Robust HotStart only needs 30 sec for activation, an initial denaturation of at least 3 min is recommended, especially for complex genomic templates.
- Do not combine the GC Buffer and KAPA Enhancer 1.
- KAPA2G GC Buffer and KAPA2G Buffer A contain $MgCl_2$ at a 1X concentration of 1.5 mM. Results may be improved by adding an additional 0.5 mM $MgCl_2$ to reactions (e.g., 0.5 µL 25 mM $MgCl_2$ per 25 µL reaction).
- The volumes of all components may be scaled down for smaller reactions. However, do not use less than 10 ng complex, genomic DNA or 1 ng less complex DNA in a 10 µL reaction.
- A final extension at 72°C is only required if 3'-dA-tailing of PCR products is essential for fragment analysis or cloning.

Published by:

Roche Sequencing and Life Science
9115 Hague Road
Indianapolis, IN 46256

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MC-US-07380 APP311002 A520 7/20