Key features

Fast Multiplex PCR with KAPA2G Fast HotStart allows for reductions of 30% – 70% in total reaction time.

The unique KAPA2G Fast HotStart enzyme and buffer system offers improved performance in terms of specificity and sensitivity.

Use 1.5x KAPA2G Buffer A or M and 1 unit KAPA2G Fast HotStart DNA Polymerase per 25 µL Multiplex PCR reaction.

Straightforward process for conversion or optimization of Multiplex PCR assays.

Multiplex PCR is a challenging application that typically requires more optimization than standard, single-amplicon PCR assays.

The key to successful Multiplex PCR is the ability to define a single set of reaction parameters (reagent concentrations and cycling parameters) that allows for all primers to anneal with high specificity to their target sequences and be extended with the same efficiency. Primer design, as well as the enzyme and buffer system, are critical factors in this challenge.

Introduction

The KAPA2G Fast HotStart offers faster and more efficient Multiplex PCR than competitor enzymes based on wild-type *Taq*. KAPA2G Fast HotStart DNA Polymerase is an engineered DNA Polymerase designed specifically for high performance Fast PCR. In the HotStart formulation, the enzyme is combined with a proprietary antibody that inactivates the enzyme until the initial denaturation step. This eliminates spurious amplification products resulting from non-specific priming events during reaction setup and initiation, and increases overall reaction efficiency. Together with a uniquely formulated buffer, KAPA2G Fast HotStart is the enzyme of choice for Multiplex PCR.

Key performance advantages are the following:

- Total reaction times 30% – 70% shorter than protocols based on wild-type *Taq* or hot start formulations thereof.
- A uniquely formulated buffer that improves annealing specificity, resulting in more even amplification of target fragments.
- Improved sensitivity, as a result of higher reaction efficiency. This means less template is required.
- A straightforward process for conversion or optimization of existing Multiplex PCR assays.

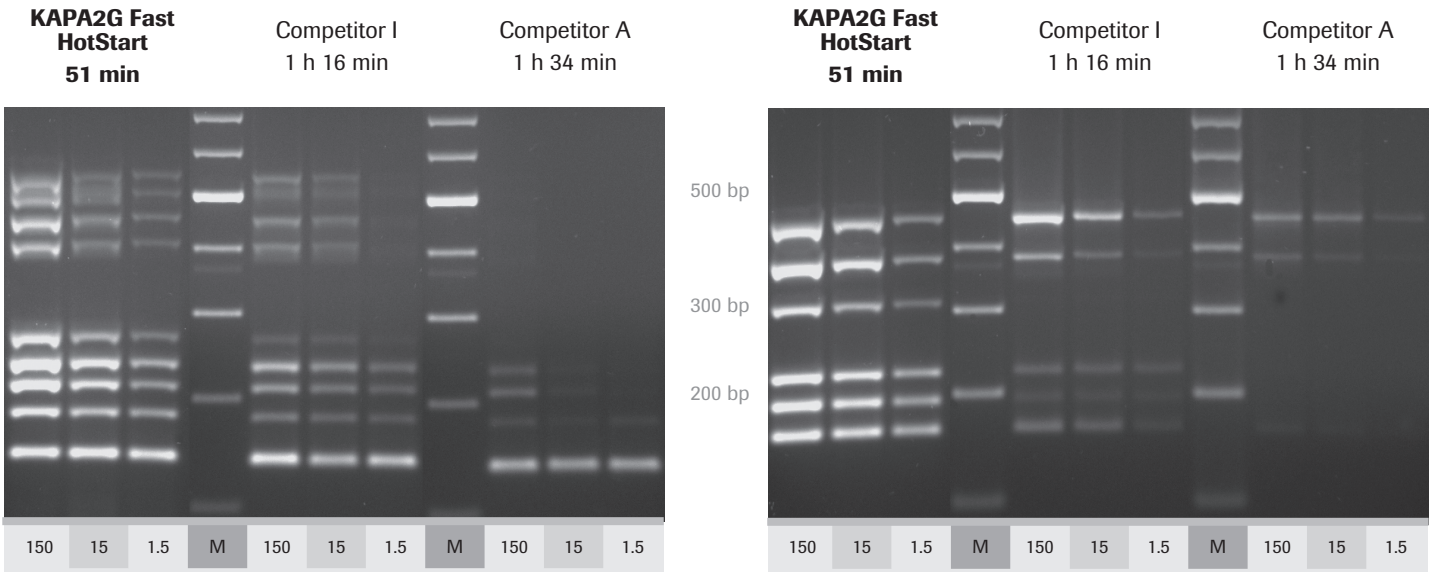

Figure 1. Amplification of 9 fragments of the human Duchenne muscular dystrophy (DMD) gene (left) and 6 fragments of the cystic fibrosis transmembrane receptor (CFTR) gene (right) in a Multiplex PCR using KAPA2G Fast HotStart or competitor hot start *Taq* formulations. Reactions (25 µL) contained 150 ng, 15 ng or 1.5 ng male human genomic DNA (as indicated below each lane) and 1 unit enzyme. KAPA2G Fast HotStart reactions were performed in 1.5x KAPA2G Buffer A and competitor reactions 1x reaction buffer. The optimal $MgCl_2$ and relative primer concentrations and annealing temperature for each assay were determined using the strategy outlined in **Optimization**. Cycling was performed with a G-Storm GS1 thermocycler (ramp rates set at 1.5ºC heating and cooling), using 3-step cycling profiles (30 cycles) with 15 sec denaturation (95ºC) and 30 sec annealing per cycle. For KAPA2G Fast HotStart, the extension time (72ºC) was 10 sec per cycle, whereas 1 min per cycle was used for competitor enzymes. The initial denaturation/enzyme re-activation time was 2 min (95ºC) for KAPA2G Fast HotStart and Competitor I and 10 min for Competitor A. A final extension step (72ºC) of 10 min was only included in Competitor A reactions. The total reaction time for each enzyme is as indicated.

Fragments amplified in each assay are (from top to bottom):
DMD – 547, 507, 459, 408, 271, 238, 213, 181, and 139 bp and CFTR – 454, 380, 309, 220, 190, and 178 bp.

Reaction setup and cycling parameters

Use the following reaction setup and cycling parameters as a starting point when setting up Multiplex PCRs with KAPA2G Fast HotStart:

Reaction component	Final concentration	Per 25 µL rxn
PCR grade water		Up to 25.0 µL
5x KAPA2G Buffer A or M*	1.5x	7.5 µL
dNTP mix (10 mM each dNTP)	0.2 mM each dNTP	0.5 µL
$MgCl_2$*	To be determined	As needed
Each forward primer (10 µM)	0.2 µM each	0.5 µL each
Each reverse primer (10 µM)	0.2 µM each	0.5 µL each
Template DNA	15 – 150 ng per 25 µL rxn**	
KAPA2G Fast HotStart DNA Polymerase (5 U/µL)	1 unit per 25 µL rxn	0.2 µL

Cycling step	Temperature and time
Initial denaturation	2 min at 95ºC
Denaturation	15 sec at 95ºC
Annealing	30 sec at optimal Ta
Extension	10 sec at 72ºC for fragments ≤1 kb; 30 sec/kb at 72ºC for fragments >1 kb
No. of cycles	30
Final extension	Not required

*5x KAPA2G Buffer A (KB5519, KB5520) contains 7.5 mM $MgCl_2$, i.e., 2.25 mM $MgCl_2$ when used at 1.5x in a Multiplex PCR. If the optimal $MgCl_2$ concentration for a specific assay is less than 2.25 mM or is unknown, use KAPA2G Buffer M (KB5528, KB5529) which is identical to Buffer A, but does not contain $MgCl_2$. See **Optimization** for details on how to determine the optimal $MgCl_2$ concentration for a specific assay.

**Depends on template complexity. For genomic templates, start with 150 ng DNA per reaction. For less complex templates (e.g., plasmid or lambda DNA), start with 15 ng per reaction.

Optimization

The following strategy is recommended to optimize an existing or new Multiplex PCR assay with KAPA2G Fast HotStart:

1. Set up a series of replicate 25 µL reactions, following the outlined guidelines. For existing assays, include $MgCl_2$ at the previously determined optimal final concentration. For new assays, start with a final $MgCl_2$ concentration of 1.5 mM. Perform an annealing temperature gradient PCR using the cycling parameters recommended above. Select the annealing temperature that yields the most even amplification of all the target fragments.
2. If even amplification of all target fragments was not obtained, repeat the experiment with several series of replicates, using a different final $MgCl_2$ concentration for each series (1.5 mM to 5 mM in 0.5 mM increments is recommended). From these results select the most optimal $MgCl_2$ and annealing temperature for the assay.
3. If some fragments are still amplified more efficiently than others, repeat the experiment with the selected optimal $MgCl_2$ and annealing temperature, but adjust the final concentration of the different primer sets in the multiplex. For fragments that are not amplified efficiently, increase the final concentration of both the forward and reverse primer for that amplicon increments of 0.1 µM, until even amplification of all fragments are obtained. For longer fragments, this adjustment is normally required.
4. If a specific fragment consistently fails to amplify, amplify it on its own using a positive control template and the same reaction setup and cycling parameters as for the Multiplex PCR. DO NOT optimize single amplicon reactions. If the problem fragment amplifies well on its own, repeat the reaction (with the Multiplex parameters), adding one additional primer set at a time. This strategy will identify primer sets that will never work well together in a Multiplex PCR—it is recommended that problematic primers are redesigned.
5. Once acceptable results are obtained, the reaction may be optimized further by systematically reducing template concentration. The number of cycles required for an optimal result may also be optimized further.
6. For “difficult” templates, such as templates with a high GC content, add 5% – 7.5% DMSO in reactions.

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