


KAPA2G™ Fast PCR Kit

1. Production Description

The KAPA2G Fast PCR Kit is designed for the conversion of existing PCR assays into Fast PCR assays for the purpose of reducing the total reaction time by up to 70%, without sacrificing reaction performance or requiring specialized PCR consumables or thermocyclers.

The kit is based on KAPA2G Fast DNA Polymerase, a second-generation enzyme that was derived from the DNA polymerase I of *Thermus aquaticus* through a process of molecular evolution. KAPA2G Fast DNA polymerase was specifically engineered for higher processivity and significantly faster extension rates than wild-type Taq polymerase. The KAPA2G Fast PCR Kit therefore offers Fast PCR that is not merely based on the reduction of cycling times, but on the intrinsic ability of the KAPA2G Fast DNA Polymerase to synthesize DNA at a much faster rate.

DNA fragments generated with the KAPA2G Fast PCR Kit have the same characteristics as DNA fragments generated with wild-type Taq polymerase and may be used for any routine downstream analysis or application, including restriction enzyme digestion, cloning and sequencing. Like wild-type Taq, KAPA2G Fast DNA Polymerase has 5'→3' polymerase and 5'→3' exonuclease activities, but no 3'→5' exonuclease (proofreading) activity. The fidelity of KAPA2G Fast DNA polymerase is similar to that of wild-type Taq. PCR products generated with KAPA2G Fast DNA polymerase are A-tailed and may be cloned into TA cloning vectors.

2. Applications

The KAPA2G Fast PCR Kit enables the fast and efficient amplification of amplicons up to 5 kb from simple and complex templates in reaction volumes of <5-25 µl. KAPA2G Fast DNA Polymerase can be used in place of wild-type Taq for most applications. For amplicons ≤1 kb, as little as 1 target copy may be used. For >1-2 kb amplicons, at least 10² copies and for >2-5 kb amplicons a minimum of 10³ copies should be used to ensure satisfactory yields and reproducible results. The kit is compatible with any conventional Peltier-based thermocycler and thin-walled PCR tubes or plates.

The conversion of existing PCR assays to Fast PCR assays is not recommended for:

- Reaction volumes >25 µl.
- Amplification of long fragments (>1 kb) from low (<10²) target copy numbers.
- PCR assays involving primers that are prone to non-specific amplification (even after reaction optimization).
- Optimized assays that give low yields of the desired amplicon despite a high target copy number (e.g. amplification from difficult templates or templates containing PCR inhibitors).
- Complex PCR assays, e.g. multiplex PCRs or PCRs involving the incorporation of nucleotide analogs.

Kit components	Product codes			
	KK 5008	KK 5010	KK 5009	KK 5011
KAPA2G Fast DNA polymerase (5 U/µl)	100 U	100 U	250 U	250 U
MgCl ₂ (25 mM)	1.6 ml	1.6 ml	1.6 ml	1.6 ml
dNTP mix (10 mM each)	250 µl	250 µl	250 µl	250 µl
5x KAPA2G Buffer A* without loading dye	1.6 ml	-	3.2 ml	-
5x KAPA2G Buffer B* without loading dye	1.6 ml	-	3.2 ml	-
5x KAPA2G Buffer A* with loading dye	-	1.6 ml	-	3.2 ml
5x KAPA2G Buffer B* with loading dye	-	1.6 ml	-	3.2 ml

*All 5x KAPA2G buffers contain MgCl₂ at a 1x concentration of 1.5 mM.

Storage

Store all components at -20 °C.

Quick Notes

- Compatible with any existing end-point PCR assay for single amplicons up to 5 kb.
- KAPA2G Fast DNA Polymerase allows for 20-70% savings in reaction time by reducing extension times.
- Use 10-20 sec/kb for 2-5 kb amplicons, 7.5-15 sec/kb for 1-2 kb amplicons, and 1-5 sec/kb for amplicons <1 kb.
- Use at least 1,000 target copies for 2-5 kb amplicons, >100 copies for 1-2 kb amplicons and >1 copy for amplicons <1 kb.
- Use Buffer A for amplicons up to 2 kb and Buffer B for larger amplicons.
- Use 0.5 units KAPA2G Fast DNA polymerase per 25 µl reaction, or less for smaller volumes.
- Do not exceed 25 µl reaction volumes.

3. Quick reference guide

3.1 To convert an existing PCR assay to a Fast PCR assay with the KAPA2G Fast PCR Kit:

- Replace your existing PCR buffer and Taq polymerase with KAPA2G Buffer A or B and KAPA2G Fast DNA Polymerase. Scale reactions down to 25 µl or less.
- Add MgCl₂ to the original final concentration if more than 1.5 mM is needed.
- Keep the final concentration of all other components the same as in your original assay.
- Use at least 1,000 target copies for >2-5 kb amplicons, >100 copies for 1-2 kb amplicons, and >1 copy for amplicons <1 kb.
- Perform your assay with the following cycling profile:

For primers with an optimal annealing temperature <65°C:

Initial denaturation	95°C	30 sec - 3 min	
Denaturation	95-96°C	5-30 sec	25-35 cycles
Annealing	Optimal Ta <65°C	5-30 sec	
Extension	72°C	1 - 5 sec/kb for amplicons ≤1 kb 7.5 - 15 sec/kb for amplicons >1-2 kb 10 - 20 sec/kb for amplicons >2-5 kb	
Final extension	72°C	10 - 30 sec/kb	
HOLD	4°C	Until products are analyzed	

For primers with an optimal annealing temperature ≥65°C:

Initial denaturation	95°C	30 sec - 3 min	
Denaturation	95-96°C	5-30 sec	25-35 cycles
Annealing / Extension	65-72°C	10 - 45 sec for amplicons ≤1 kb 30 - 90 sec for amplicons >1-2 kb 90 - 150 sec for amplicons >2-5 kb	
Final extension	72°C	10 - 30 sec/kb	
HOLD	4°C	Until products are analyzed	

3.2 To optimize a Fast cycling protocol

- Reduce the extension time in each cycle to fall within above ranges.
- Once the optimal extension time has been determined, systematically reduce:
 - Initial denaturation time
 - Denaturation time in each cycle
 - Annealing time in each cycle
 - Final extension time

Tips:

- For fast ramping cyclers, complex targets and certain primers, longer denaturation and annealing times will be needed.
- On slow ramping cyclers, the denaturation and annealing times in each cycle may be shorter.



4. Understanding Fast PCR

Converting an existing PCR assay to a Fast PCR assay that requires significantly less thermocycler time is an attractive proposition, but may end up costing time and reagents if the conversion compromises reaction performance (sensitivity, yield or reproducible amplification). Before using this (or any other) Fast PCR kit, it is therefore important to understand the basic principles of Fast PCR.

4.1 Cycling processes

A PCR cycle is based on three processes: – denaturation, primer annealing and extension. The relative contribution of each of these to the total reaction time depends on a number of factors, and suboptimal execution of any step may decrease the efficiency of the reaction, leading to lower yield of the target amplicon, reduced sensitivity and/or inconsistent amplification.

After initial denaturation of the template DNA, complete denaturation of product formed in each cycle is necessary for the exponential accumulation of the target amplicon. Denaturation is typically conducted at 94-96°C and the efficiency of this step is determined by efficiency of heat transfer to the reaction components and the nature of the DNA template and fragment that is being amplified. Thin-walled PCR tubes and plates facilitate heat transfer, whereas large reaction volumes reduce its efficiency. Complex templates and amplicons with a high GC-content and/or stable secondary structure usually require longer denaturation times and/or higher temperatures to ensure that the maximum number of template molecules are available during each successive cycle.

Exponential accumulation of new amplicon molecules depends on the efficient annealing of primers to each available template molecule during each successive PCR cycle. Complex templates (e.g. genomic DNA) require longer annealing times than simple ones (e.g. plasmid, phage or bacmid DNA), and certain primer types (e.g. those with a low GC-content) may require longer annealing times. The effective annealing time is determined by the characteristics of the particular primer pair. Primers with a broad annealing temperature range result in longer effective annealing times than primers with sharply defined optimal annealing temperatures (see Sections 4.2 and 4.3 for more details).

Exponential accumulation of the desired amplicon finally depends on full extension of each annealed primer molecule by the DNA polymerase. Conventional protocols with Taq DNA polymerase provide for 1 min/kb extension time at the optimal temperature for Taq polymerase activity (72°C). The relative contribution of extension time to the total reaction time increases with increasing amplicon size (Table 1).

Table 1: Total cycle extension time as a percentage of total reaction time for different amplicon lengths in a conventional PCR assay with wild-type Taq

Amplicon length	Extension time per cycle	Total reaction time	Total extension time	% of total reaction time
500 bp	0.5 min	69.4 min	17.5 min	25.2%
1 kb	1.0 min	87.2 min	35.0 min	40.1%
2 kb	2.0 min	122.7 min	70.0 min	57.0%
3 kb	3.0 min	158.2 min	105.0 min	66.4%
4 kb	4.0 min	193.7 min	140.0 min	72.3%
5 kb	5.0 min	229.2 min	175.0 min	76.4%

Figures are based on a 35-cycle reaction profile consisting of an initial denaturing step of 2 min at 95°C; 15 sec denaturing (95°C), 15 sec annealing (60°C) and 1 min/kb extension (72°C) per cycle, and a final extension at 72°C of 30 sec/kb. Thermocycler ramp rates of 2°C/sec heating and 1°C/sec cooling were used in calculations.

The amount of time that can be saved by using competitor Fast PCR kits and protocols based on wild-type Taq is limited by the extension rate of the enzyme. To achieve shorter assay times the denaturation and annealing times in each cycle have to be shortened. Special ultra-thin plastics and reduced reaction volumes allow for improved heat transfer during denaturation, and specially formulated buffers are employed to increase the efficiency of primer annealing, thereby reducing annealing times. In addition, extension times are shortened, particularly for short amplicons. However, such “artificially” shortened reaction profiles often result in reduced efficiency of one or more of the cycling processes, thereby reducing the yield of the target amplicon and/or sensitivity of the assay. Results comparable to those obtained with the original assay are consequently only achievable in assays with short amplicons and very high target copy numbers.

In contrast, the KAPA2G Fast PCR Kit is based on an engineered polymerase with an intrinsic ability to synthesize DNA faster than wild-type Taq. The protocols outlined in Section 6 are therefore primarily based on reduced extension times that allow for a reduction of 20% to more than 70% of the original PCR time without the risk of compromising reaction performance.

4.2 Fast vs. slow thermocyclers

The time it takes to complete the same cycling profile on different thermocyclers differs significantly and this “real” or elapsed time (vs. programmed time) is determined by the thermal ramping rates of individual instruments (Table 2). The majority of conventional (Peltier-based) thermocyclers are only capable of heating the block at 1-1.5°C/sec. Cooling is usually slower and rates of less than 1°C/sec are typical. Silver and gold (plated) blocks used in fast ramping instruments developed in recent years are capable of heating rates of 3-6°C/sec and maximum cooling rates of just more than 4°C/sec.



Table 2: Approximate assay times for the amplification of different sized amplicons with wild-type Taq using thermocyclers with different thermal ramping rates

	Fast ramping	Medium ramping	Slow ramping
Heating Cooling	6°C/sec 4°C/sec	3°C/sec 1.5°C/sec	1.5°C/sec 0.75°C/sec
500 bp	46 min	59 min	1 h 27
1 kb	1 h 04	1 h 16	1 h 45
2 kb	1 h 39	1 h 52	2 h 20
3 kb	2 h 15	2 h 27	2 h 56
4 kb	2 h 50	3 h 03	3 h 31
5 kb	3 h 26	3 h 38	4 h 05

Figures are based on a 35-cycle reaction profile consisting of an initial denaturing step of 2 min at 95°C; 15 sec denaturing (95°C), 15 sec annealing (60°C) and 1 min/kb extension (72°C) per cycle, and a final extension at 72°C of 30 sec/kb.

The KAPA2G Fast PCR Kit may be used in conjunction with any conventional thermocycler irrespective of ramp rates. The same assay will take longer to complete on a slow ramping than on a fast ramping instrument. The relative amount of time saved by converting your existing assay into a Fast assay using this kit is typically between 20 and >60%, for slow ramping instruments and between 35 and >70% for when fast ramping cyclers are used (see Section 6 for more details).

When converting your existing assay to a Fast assay, or designing a new Fast assay, it is important to take the thermal ramping characteristics of your specific thermocycler into account, as the “real” time spent on each process in each cycle (vs. the programmed time) is longer on slower ramping instruments.

For example, for a fast ramping instrument (+6°C/sec; -4°C/sec), the time spent to cool the instrument down between denaturing at 95°C and annealing at 60°C is 8.75 sec, whereas it takes almost 47 sec to achieve the same on a slow ramping (+1.5°C/sec; -0.75°C/sec) cycler. Part of this cooling time will contribute to template denaturation, and part to primer annealing. For heating the block between the annealing (60°C) and extension (72°C) steps, the times are approximately 3.8 and 8 sec for the same fast and slow instruments, respectively. Because of this “extra” time added to each of the cycling processes in each cycle, the programmed times for slower ramping instruments may be slightly shorter than those for fast ramping cyclers (see Section 6 for detailed cycling profiles). For the same programmed times, the “real” cycle times of slower ramping instruments often translate into higher yields and/or sensitivity, particularly for long amplicons.

4.3 Primer design and assay times

Primer design is a significant factor in Fast PCR. Guidelines for optimal primer design are widely available in printed and online resources and should be followed as far as possible. Different algorithms are available for calculating the melting temperature (T_m) of a primer and an annealing temperature (T_a) 3-5°C than the T_m is usually a good first approximation. Primer design programs and T_m calculators are, however, unable to predict the behavior of a particular primer pair with a particular template under specific reaction conditions, namely whether the primer pair will anneal over a broad T_a range or display a sharp optimal T_a. A T_a gradient PCR is therefore recommended as the first step in the optimization of any PCR assay, particularly those that are to be converted to Fast assays.

Primers with a sharply defined optimal T_a are used in traditional 3-step cycling profiles (where each cycle is programmed with a specific time for denaturation, annealing and extension). Primers that anneal efficiently over a broad T_a range (particularly in the range of 65-72°C) may, however, be used in 2-step cycling profiles, where the annealing and extension step is combined into a single process and each cycle consists of only two specified times. When converting an existing PCR assay to a Fast assay, a significant amount of reaction time may be saved if the specific primer pair is suitable for a 2-step protocol. This should be taken into consideration when new assays are designed. More details about 2- and 3-step cycling profiles and the conversion of existing cycling profiles are given in Section 6.

5. Fast PCR with the KAPA2G Kit: Reaction setup

5.1 Converting an existing PCR assay to a Fast assay

Any existing PCR assay with which a satisfactory yield of the target fragment is routinely obtained may be converted to a Fast assay using the KAPA2G Fast PCR Kit, by following the guidelines below.

The basic steps:

Simply substitute your current PCR buffer and polymerase with the appropriate KAPA2G buffer and KAPA2G Fast DNA Polymerase. Two 5x KAPA2G buffers are provided. Buffer A is usually recommended for amplicons up to 2 kb and buffer B for >2-5 kb amplicons. During this conversion, keep the following in mind:



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- If your original protocol was based on reaction volumes >25 µl, reduce the amounts of all reagents (except template DNA – see below) proportionally to a reaction volume of 25 µl or less.

When using 96-well PCR plates, reaction volumes of less than 10 µl are not recommended, as evaporation of reaction mixes (especially from wells along the edge of the plate) often occur. This affects the outcome of low volume reactions more severely than higher volume reactions.

- When reducing reaction volumes, the amount of template DNA should be calculated on the basis of target copy number. For amplicons ≤1 kb, as little as 1 copy may be used. For >1-2 kb amplicons, do not use less than 10² copies and for >2-5 kb amplicons, use at least 10³ copies to ensure satisfactory yields and reproducible results.

Always use the highest possible target copy number without exceeding 10 ng/µl reaction volume for complex templates (genomic DNA) or 1 ng/µl reaction volume for simple templates (e.g. plasmid DNA). The approximate target copy number for any template may be calculated using the following equation:

$$(M \times 1,515) / bp \times (6.022 \times 10^{11}) \times P$$

Where M = mass in µg of template DNA in the reaction, bp = number of base pairs of total template (not target) DNA and P = number of priming sites of primer pair on template. For example: the target copy number for a single copy gene in 1 ng human genomic DNA equals: $(1 \times 10^{-3}) \times 1,515 / (3.3 \times 10^9) \times (6.022 \times 10^{11}) \times 1 = 280$ copies.

- KAPA2G PCR buffers A and B both contain MgCl₂ at a 1x concentration of 1.5 mM. If your original protocol requires a higher final MgCl₂ concentration, add the appropriate volume of 25 mM MgCl₂ supplied in the kit to each reaction mix.
- Use primers at a final concentration of 0.5 µM each.
- If your assay requires any other component (e.g. BSA or DMSO) for optimal performance, include this to the same final concentration as in your original assay.

Finally, convert your original cycling profile to a Fast cycling profile according to the guidelines outlined in Section 6.

If further optimization is required:

If the basic conversion steps do not yield the desired result (i.e. an insufficient yield of the target fragment or significant non-specific amplification), optimize your reaction by trying one or more of the following:

- Use KAPA2G buffer A instead of B or *vice versa*.
- Increase the amount of template per reaction if possible.
- Perform a Ta gradient PCR to ensure that the optimal Ta for your primer pair is being used.
- Take care to set reaction mixes up on ice and perform a manual hot start by keeping mixes on ice and only placing them in the thermocycler once the lid is fully heated and the block has reached denaturation temperature.
- Optimize the fast cycling profile initially selected by following the guidelines in Section 6.1.

5.2 Designing a new Fast PCR assay

Fast PCR assays do not only have to be converted from existing assays, but may also be designed from first principles using the KAPA2G Fast PCR Kit and guidelines below:

- Design a set of primers by following the general guidelines for primer design. A primer pair with a Ta in the range of 65-72°C is preferable, as this will allow for the fastest 2-step cycling profile to be used.
- Make a master mix for 12 x 20 µl reactions, containing the following components (always prepare 10% more master mix than needed):

	Final concentration	20 µl
PCR grade water		Up to 20.0 µl
5x KAPA2G Buffer A or B*	1x	4.0 µl
MgCl ₂	(1.5 mM in buffer)	-
dNTP mix (10 mM each)	0.2 mM each dNTP	0.4 µl
Forward primer (10 mM)	0.5 µM	1.0 µl
Reverse primer (10 mM)	0.5 µM	1.0 µl
Template DNA	≤200 ng per 20 µl rxn for complex genomic DNA ≤20 ng per 20 µl rxn for less complex templates (e.g. plasmid, lambda)	>1 copy for amplicons ≤1 kb >10 ² copies for amplicons >1-2 kb >10 ³ copies for amplicons >2-5 kb
KAPA2G Fast DNA Polymerase (5 units/µl)	0.4 units/20 µl rxn	0.08 µl

*Buffer A is recommended for amplicons up to 2 kb and buffer B for >2-5 kb amplicons. However, the inverse may be optimal for your specific primer-template combination and it is recommended that the experiment be performed in duplicate to determine the best buffer for the new assay.



c. Split the master mix (for each buffer) into 12 thin-walled PCR tubes and perform a Ta gradient PCR using the following 3-step cycling profile:

Initial denaturation	95°C	2 min	
Denaturation	95°C	30 sec	35 cycles
Annealing	52-72 °C (in 12 increments)	30 sec	
Extension	72°C	10 sec/kb for amplicons ≤1 kb 15 sec/kb for amplicons >1-2 kb 20 sec/kb for amplicons >2-5 kb	
Final extension	72°C	30 sec/kb	
HOLD	4°C	Until products are analyzed	

The results from this experiment will indicate:

- The optimal Ta for the primer pair in this assay.
- Whether the primer pair is suitable for use in a fast 2-step protocol (only if efficient amplification was obtained in the Ta range of 65-72°C).
- Whether further optimization of the reaction components is required to maximize yield of the target fragment. This will only be necessary if the yield of the target fragment was very low and/or significant non-specific amplification was obtained across the entire Ta range.

d. Optimize the assay (if needed) by repeating the assay at the most optimal Ta determined in the above experiment. The following may be attempted:

- To improve yield: use more template per reaction.
- To reduce non-specific amplification:
 - Perform a MgCl₂ gradient experiment (1.5 to 5 mM final concentration of MgCl₂ in 0.5 mM increments) to establish the optimal MgCl₂ concentration for the assay *and/or*
 - Reduce the final concentration of each primer in the reaction mix (but do not less than 0.1 uM of each primer) *and/or*
 - Redesign the primers to increase their length and/or reduce intra- and inter-primer annealing and/or secondary priming sites in the template

e. Once assay components have been optimized, optimize the cycling profile by following the guidelines in Section 6, to determine the shortest total reaction time that may be used without compromising the yield of the target fragment.

6. Fast PCR with the KAPA2G Kit: Cycling profiles

6.1 Converting an existing 3-step PCR assay to a Fast 3-step assay

Basic conversion: simply reduce cycle extension times.

The simplest way to convert an existing, optimized PCR assay employing wild-type Taq polymerase to a Fast assay with KAPA2G Fast DNA Polymerase is to retain the original cycling profile (including the number of cycles) but reduce the extension time in each cycle as outlined below. Perform a series of parallel experiments, in which the extension rate is systematically decreased by the specified increment, to determine the shortest optimal extension time per cycle for the assay.

Amplicon size (kb)	Start with	Reduce in increments of	End with
≤1 kb	5 sec/kb	1 sec/kb	1 sec/kb
>1-2 kb	15 sec/kb	2.5 sec/kb	7.5 sec/kb
2-5 kb	20 sec/kb	2.5 sec/kb	10 sec/kb

Extension time per cycle = extension rate (sec/kb) x amplicon length in kb

By simply reducing the extension time in each PCR cycle, a reduction of 20-70% in the total reaction time is achievable by using KAPA2G Fast DNA Polymerase instead of wild-type Taq (see Table 3).



Table 3: Examples of savings in total PCR assay times achievable by using KAPA2G Fast DNA polymerase instead of wild-type Taq*

Amplicon size	KAPA2G Fast extension rate	Total assay time with KAPA2G	Total assay time with wild-type Taq**	% time saved with KAPA2G Fast PCR kit
Fast ramping cyclers (+6°C/sec; -4°C/sec)				
500 bp	1 sec/kb	28.8 min	46.1 min	38%
1 kb	1 sec/kb	29.3 min	64.0 min	54%
2 kb	7.5 sec/kb	37.7 min	98.9 min	62%
3 kb	10 sec/kb	46.7 min	134.2 min	65%
5 kb	10 sec/kb	58.8 min	204.7 min	71%
Slow ramping cyclers (+1.5°C/sec; -0.75°C/sec)				
500 bp	1sec/kb	62.8 min	80.0 min	22%
1 kb	1 sec/kb	63.2 min	97.6 min	35%
2 kb	7.5 sec/kb	71.6 min	132.9 min	46%
3 kb	10 sec/kb	80.6 min	168.1 min	52%
5 kb	10 sec/kb	92.8 min	238.6 min	61%

*Figures are based on a 35-cycle reaction profile consisting of an initial denaturing step of 2 min at 95°C; 15 sec denaturing (95°C) and 15 sec annealing (60°C) per cycle and a final extension at 72°C of 30 sec/kb.

**Based on 1 min/kb used in conventional protocols for all amplicon lengths.

Reducing the total reaction time further

Once the optimal extension rate has been established, it may be possible to reduce the total reaction further by systematic optimization of the other cycling times in your assay, as outlined below:

a. Optimize the initial template denaturation time:

KAPA2G Fast DNA Polymerase does not require an initial re-activation step and initial template denaturation for as little as 30 sec at 95°C is required for simple templates. For more complex, genomic DNA or templates with a high GC-content, an initial denaturation of 1-3 min at 95°C is sufficient.

b. Optimize the denaturation time in each cycle:

If your original protocol is based on a denaturing time of >30 sec per cycle, it may be possible to save more time by shortening the programmed denaturation time in each cycle. The optimal time for a particular assay will depend on the reaction volume, PCR plastics used, nature of the template and fragment being amplified, target copy number, amplicon length and thermal ramping rates of the thermocycler.

To determine the shortest optimal programmed denaturation time per cycle (without reducing the overall assay efficiency), perform your assay with the original programmed annealing time and optimal extension time for KAPA2G Fast Polymerase determined in the above experiment, but reduce the original cycle denaturation time in increments of 5 sec (down to 10 sec and thereafter in increments of 1 sec). The shortest programmed denaturation time that does not result in a reduction of amplicon yield is optimal.

c. Optimize the annealing time:

If your original protocol is based on an annealing time of >30 sec per cycle, it may be possible to save more time by shortening the programmed annealing time in each cycle. The optimal time for a particular assay will depend on the reaction volume, PCR plastics used, nature of the template and fragment being amplified, target copy number, amplicon length, primer characteristics and thermal ramping rates of the thermocycler.

To determine the shortest optimal programmed annealing time per cycle (without reducing the overall assay efficiency), perform your assay with the optimal denaturation and extension times for KAPA2G Fast polymerase determined in the above experiments, but reduce the original cycle annealing time in increments of 5 sec (down to 10 sec and thereafter in increments of 1 sec). The shortest programmed annealing time that does not result in a reduction of amplicon yield is optimal.



d. Optimize the final extension time:

A final extension step of several minutes at 72°C is traditionally included in PCR protocols for the purpose of completing any incomplete fragments and generation of 3'-dA overhangs. This final extension step may be reduced to 10–60 sec per kb for the fragment amplified. If you are uncertain and do not want to spend time optimizing this step, include a final extension of 30 sec/kb in your assay.

e. Optimize the cycle number:

The number of cycles required to produce a satisfactory yield of the target amplicon mainly depends on the nature of the template (complex or simple), amplicon length and target copy number. Apply the following simple guidelines as a starting point for any assay, irrespective of template complexity or amplicon length:

Target copy number	Number of cycles
≤10 ⁴	35
10 ⁴ - 10 ⁶	30
>10 ⁶	25

By following the above steps, the total assay time based on fast extension times with KAPA2G Fast Polymerase may be reduced **by another 15-60%**, allowing for the efficient routine amplification of short amplicons (<1 kb) in less than 20 min on fast ramping cyclers.

6.2 Converting an existing 3-step PCR assay to a Fast 2-step assay

A standard 3-step cycling profile may be converted to a Fast 2-step profile by combining the annealing and extension processes in each cycle. Instead of spending a programmed period of time at 72°C in each cycle, the profile cycles between the denaturation temperature (95-96°C) and the combined annealing/extension temperature, spending a fixed period of time at each of these.

Primer pairs with a sharply defined optimal Ta <65°C are not recommended for 2-step cycling profiles, as no time is saved by simply combining the annealing and extension steps. To retain full efficiency with such primer pairs, the combined annealing/extension time often has to be longer than the sum of the times for the individual steps in the 3-step profile, to compensate for the fact that polymerases are slower at lower temperatures.

The real advantage of 2-step cycling profiles lies in the fact that less time is spent heating and cooling the samples when primers operate efficiently at a temperature where polymerase activity is not significantly reduced (65-72°C). If the primer pair for your assay displays this characteristic, a significant amount of time may be saved by converting your original 3-step profile to a 2-step profile as indicated in the table below:

Initial denaturation	95°C	2 min	
Denaturation	95-96°C	Start with time in original 3-step profile	35 cycles
Annealing / Extension	65 - 72°C*	Start with: 45 sec for amplicons ≤1 kb 90 sec for >1-2 kb amplicons 150 sec for >2-5 kb amplicon	
Final extension	72°C	30 sec/kb	
HOLD	4°C	Until products are analyzed	

*Use highest combined temperature at which primer pair performs efficiently.



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Start with the combined annealing/extension time for each amplicon length given in the table. If satisfactory results are obtained, optimize (reduce) the total reaction time further by:

- a. Reducing the combined annealing/extension time per cycle further, until the shortest programmed time that does not reduce reaction efficiency (yield or sensitivity) is determined. This is done by performing duplicate experiments in which the annealing/extension time is systematically reduced with the increments indicated below:

Amplicon size (kb)	Start with	Reduce in increments of	End with
≤1 kb	45 sec	5 sec	10 sec
>1-2 kb	90 sec	10 sec	30 sec
2-5 kb	150 sec	15 sec	90 sec

- b. Reduce the initial denaturation time if possible (see Section 6.1a for details)
- c. Reduce the denaturation time in each cycle if possible (see Section 6.1b for details)
- d. Reduce the final extension time if possible (see Section 6.1d for details)
- e. Reduce the number of cycles – for simple templates only (see Section 6.1e).

6.3 Setting up a Fast PCR assay from first principles using the KAPA2G Fast PCR Kit

If your assay has been newly designed (according to the guidelines in Section 5.2), determine the optimal fastest cycling profile as follows:

For 3-step primers, start with:

Initial denaturation	95°C	2 min	
Denaturation	95-96°C	30 sec	35 cycles
Annealing	Optimal Ta <65°C*	30 sec	
Extension	72°C	10 sec/kb for amplicons ≤1 kb 15 sec/kb for amplicons >1-2 kb 20 sec/kb for amplicons >2-5 kb	
Final extension	72°C	0 - 30 sec/kb	
HOLD	4°C	Until products are analyzed	

*As determined in a Ta gradient experiment (Section 5.2)

For 2-step primers, start with:

Initial denaturation	95°C	2 min	
Denaturation	95-96°C	30 sec	35 cycles
Annealing / Extension	65-72°C	45 sec for amplicons ≤1 kb 90 sec for amplicons >1-2 kb 150 sec for amplicons >2-5 kb	
Final extension	72°C	0 - 30 sec/kb	
HOLD	4°C	Until products are analyzed	

*Use highest combined temperature at which primer pair performs efficiently.

Now reduce the total reaction time further by:

- a. Optimizing the extension time (for 3-step profiles) or combined annealing/extension time (2-step profiles). This is done by performing parallel assays in which the (annealing/) extension time in each cycle is systematically decreased by the increments specified below, to determine the shortest optimal (annealing/) extension time per cycle for the assay:

For 3-step profiles:

Amplicon size (kb)	Start with	Reduce in increments of	End with
≤1 kb	5 sec/kb	1 sec/kb	1 sec/kb
>1-2 kb	15 sec/kb	2.5 sec/kb	7.5 sec/kb
2-5 kb	20 sec/kb	2.5 sec/kb	10 sec/kb

Extension time per cycle = extension rate (sec/kb) x amplicon length in kb

For 2-step profiles:

Amplicon size (kb)	Start with	Reduce in increments of	End with
≤1 kb	45 sec	5 sec	10 sec
>1-2 kb	90 sec	10 sec	30 sec
2-5 kb	150 sec	15 sec	90 sec

Combined annealing/extension time per cycle = time given in column 2

- b. Reducing the initial denaturation time if possible (see Section 6.1a for details)
- c. Reducing the denaturation time in each cycle if possible (see Section 6.1b for details)
- d. Reducing the final extension time if possible (see Section 6.1d for details)
- e. Reducing the number of cycles – for simple templates only (see Section 6.1e).



7. Troubleshooting

In addition to the recommendations in Sections 5 and 6:

No amplification product

- Repeat PCR ensuring that all reaction components have been included at the correct concentrations.
- Check that thermocycler is functioning and is programmed correctly.

Only primer-dimers visible or very low yield

- Make sure reaction volumes do not exceed 25 μ l.
- Increase the amount of template or make fresh primer and/or template dilutions.
- Lower the annealing temperature or determine the optimal annealing temperature empirically in a gradient PCR.
- Increase the denaturing, annealing and/or extension time in each cycle and/or the number of cycles.
- Make sure PCR tubes or plates are suitable for thermocycler.

Non-specific, low molecular weight bands or high molecular weight smears

- Set reactions up on ice and/or use a "manual" hot start.
- Increase the annealing temperature or determine optimal annealing temperature empirically in a gradient PCR.
- Reduce the extension time and/or number of cycles.
- Use KAPA2G Buffer B instead of Buffer A or *vice versa*.
- Optimize the $MgCl_2$ concentration in a gradient PCR.
- Determine optimal concentration of template in a template dilution series experiment.
- Review primer design.

8. Specifications

8.1 Shipping and storage

KAPA2G Fast PCR Kits are shipped on dry ice or ice packs, depending on the country of destination. Upon receipt, store the entire kit at $-20^{\circ}C$ in a constant-temperature freezer. When stored under these conditions and handled correctly, all kit components will retain full activity until the expiry date indicated on the kit.

8.2 Handling

Always ensure that all kit components are fully thawed before use. Vortex 5x KAPA2G PCR buffers after each freeze-thaw cycle. Keep components on ice during reaction setup and return to $-20^{\circ}C$ for long-term storage.

8.3 Quality control

KAPA2G Fast DNA Polymerase is extensively purified through the use of multiple chromatography steps. The final formulation contains <2% contaminating protein, as determined in an Agilent Protein 230 Assay. Each batch of enzyme, buffer and other components are subjected to stringent quality control tests, are free of contaminating exo- and endonuclease activities and meet strict requirements with respect to DNA contamination.

8.4 Product use limitations and licenses

KAPA2G Fast PCR Kits are developed, designed and sold exclusively for research purposes and *in vitro* use. Neither the product, nor any individual component, was tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals. Please refer to the MSDS, which is available on request.

Certain applications of this product are covered by patents issued to parties other than Kapa Biosystems and applicable in certain countries. Purchase of this product does not include a license to perform any such applications. Users of this product may therefore be required to obtain a patent license depending upon the particular application and country in which the product is used.



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