Fast PCR: General Considerations for Minimizing Run Times and Maximizing Throughput

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Introduction

The polymerase chain reaction (PCR) has traditionally been optimized for specificity and, to a lesser extent, product yield. The speed with which the reaction is completed has been of secondary importance. The availability of software to aid in primer and PCR product design, as well as the use of reagents that can tolerate a range of reaction conditions, has allowed researchers to focus on maximizing throughput by minimizing PCR cycling times.

Some manufacturers have recently introduced instruments and consumables that are targeted to those performing "fast PCR" — a PCR protocol completed in less than half the typical 90 min. Although many researchers assume that fast PCR is only obtainable through the purchase of these specialized, faster ramping thermal cyclers, in this article, we demonstrate that most of the time savings in fast PCR are achieved simply by modifying thermal cycling conditions.

We provide general considerations for accomplishing fast PCR without a specialized thermal cycler and demonstrate that with conventional instruments, reagents, and reaction vessels it is possible to:

- Shorten run times for standard PCR from around 90 to 35 min
- Reliably amplify long targets (1–20 kb) 3- to 4-fold faster than with standard protocols
- Obtain real-time quantitative PCR (qPCR) data with SYBR Green or TaqMan chemistries in under an hour

Saving Time at Each Step of a PCR

Standard PCR protocols for amplifying targets of less than 1,000 bp comprise several steps, each of which can be modified to shorten overall run times. Overall reaction time for conventional PCR can be reduced from about 90 min to under 35 min by shortening hold times and by minimizing the temperature differential between one step and the next (Figure 1). Some simple considerations for shortening run times are provided in the Addendum. The rationale for each of these modifications is explained below.

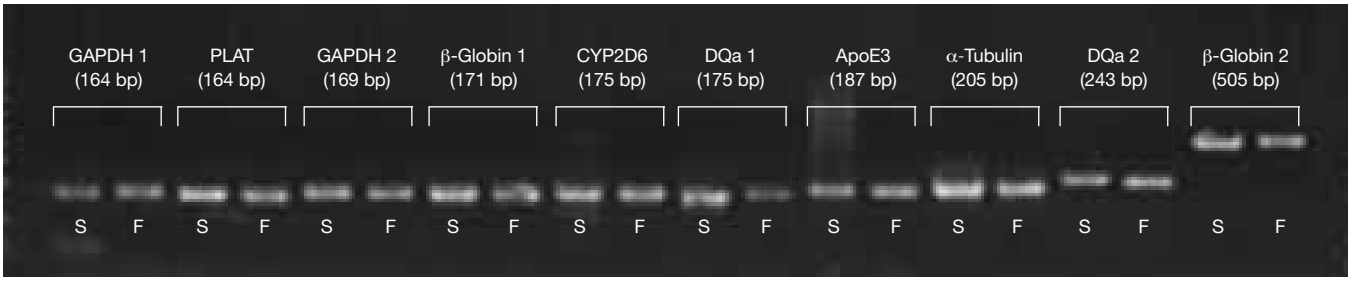

Fig. 1. Reactions run in less than 35 min generate results comparable to those run in 90 min. S, standard protocol: 95°C for 3 min, then 35 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec, followed by 72°C for 10 min. Actual run time, 88 min. F, fast protocol: 98°C for 30 sec, then 35 cycles of 92°C for 1 sec and 70°C for 15 sec, followed by 72°C for 1 min. Actual run time, 32 min. All PCR products were designed with primer T_m = 68–72°C. Each 20 µl reaction contained 2,000 human genome targets.

Initial Denaturation

The first step in the PCR is generally performed at 94–96°C for 2–20 min. This step denatures the initial template into single-stranded DNA and also activates hot-start polymerases. While 2–3 min at 94–95°C is usually sufficient to fully denature total genomic DNA, some hot-start polymerases require 15 or 20 min at 95°C to be activated. When using an antibody-modified hot-start polymerase such as iTaq™, however, both activation and initial denaturation can be accomplished in just 15–30 sec at 98°C (Figure 2). These parameters can also work well for qPCR, with no deleterious effects on reaction efficiencies or C_T values over a range of target concentrations (data not shown).

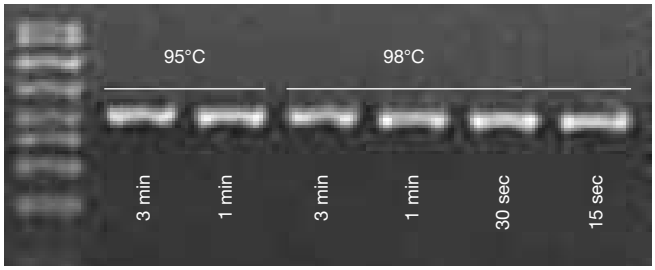

Fig. 2. Initial denaturation and enzyme activation time requires 30 sec or less with iQ supermix, which uses iTaq hot-start polymerase. Gel image shows a 505 bp β-globin target amplified using iTaq polymerase with a range of initial denaturation conditions. Protocol included initial denaturation conditions as shown, then 35 cycles of 92°C for 1 sec and 68°C for 15 sec, followed by 72°C for 1 min. Actual run time, 34–38 min.

Denaturation While Cycling

The hold times and temperatures required to denature the template during PCR cycling are not as stringent as in the initial denaturation step, because the template being denatured is a PCR product, which is usually much shorter and less complex than the initial template DNA. We have found that a 1 sec denaturation at 92°C is sufficient for a variety of PCR products amplified with iQ™ supermix, including the 83.5% GC, 505 bp PCR product in Figures 1 and 2, as well as a 64% GC, 150 bp PCR product in lambda DNA (data not shown). This is consistent with the observation of Yap and McGee (1991) that temperatures above 92°C are unnecessary for denaturing PCR products shorter than 500 bp.

Annealing and Extension

Because most polymerases are highly active in the temperature range typical for primer annealing (55–70°C), the annealing and extension steps of a PCR protocol can often be consolidated into a single step. Using a two-step PCR protocol rather than the standard three-step protocol can result in a significant reduction in run time. Further reductions can be achieved by reducing the incubation time of this combined annealing/extension step. The standard annealing times (15–60 sec) and extension times (1 min per kb of PCR product) are, in most instances, unnecessarily long. Because primer concentrations are high relative to template, annealing of primers requires just a few seconds at the optimal reaction temperature. Furthermore, a well-optimized reaction using iTaq polymerase can amplify PCR products efficiently with much shorter extension times. As shown in Figure 1, a 15 sec combined annealing/extension incubation can be sufficient for PCR products up to 500 bp. Even shorter extension times are possible with iProof™ polymerase, which can amplify a 2 kb target with an annealing/extension time under 15 sec.

It is important to optimize the annealing/extension temperature, because it is the major determinant of specificity of the reaction. If the annealing temperature is too high, the primers will not anneal efficiently, resulting in no amplification or poor yield; if it is too low, primer mismatches and nonspecific amplification may occur, and yield may be diminished (Rychlik et al. 1990). To maximize both speed and specificity, use the highest possible annealing temperature without sacrificing adequate reaction yield. Gradient-enabled thermal cyclers allow optimization of the annealing temperature in a single run.

To establish general considerations for choosing primers and annealing/extension temperatures for fast PCR, we performed a series of reactions using a range of annealing/extension temperatures and a panel of primer pairs that had average T_m values varying from 58 to 72°C. Figure 3 shows that a range of annealing/extension temperatures worked well for fast PCR. The fastest overall reaction times for each primer pair were obtained by using the highest annealing/extension temperature that generated a good band on the gel (i.e., strong intensity, single product). See the Addendum for the simple considerations derived from these experiments.

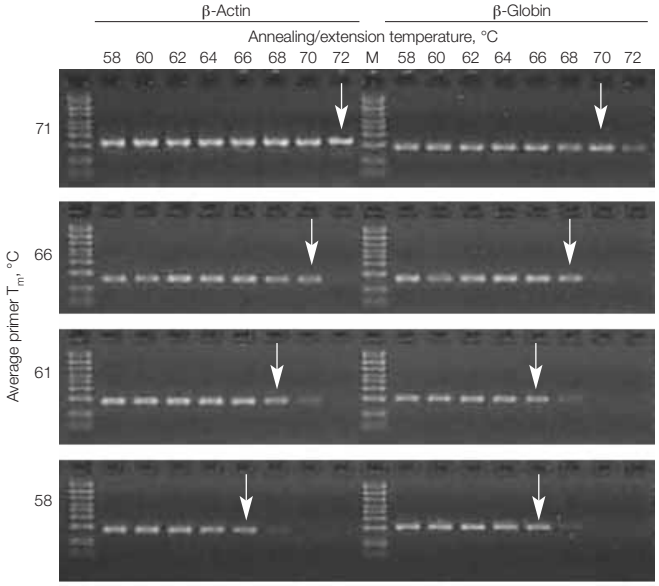

Fig. 3. Determining the optimal annealing and extension temperature for fast PCR. The cycling protocol was 98°C, 30 sec; then 35 cycles of 92°C, 1 sec and annealing/extension temperature, 15 sec; then 72°C, 1 min. Actual run time, 33–42 min. Arrows indicate the reaction conditions that provided the shortest ramp times and highest specificity while maintaining good yield.

Note: If you choose to redesign primers for faster PCR reactions, many primer design programs (e.g., Primer3 software) simplify design by allowing you to specify the desired primer T_m. Existing primers with low T_m values can often be easily adapted to faster PCR by adding 2–4 bases to the 5' ends. Naturally, such primer modifications must be checked for new self- and cross-primer complementarity.

Ramping Time

Ramping time is the time required by the thermal cycler to transition from one incubation temperature to another. Two parameters contribute to ramping time: the ramp rate of the cycler and the difference between consecutive temperatures. Smaller temperature excursions result in shorter ramping times. While the contribution of ramp rate to overall cycling time has been highlighted by manufacturers of faster-ramping thermal cyclers, the time saved by using these specialized cyclers is relatively minor (6–8 min) compared to the savings gained from optimizing thermal cycling parameters for speed (55–65 min; see Figure 4).

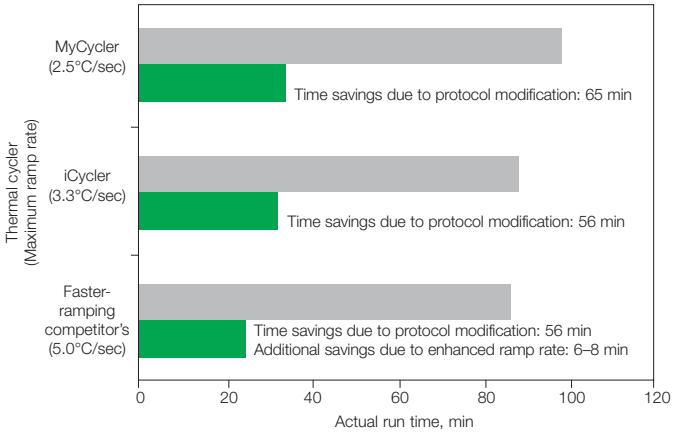

Fig. 4. Run time savings from protocol modification vs. faster ramping. A standard (■ , three-step) protocol and a modified fast (■ , two-step) protocolwere run on three thermal cyclers with differing ramp rates — the iCycler® (maximum ramp rate, 3.3°C/sec), the MyCycler™ (2.5°C/sec), and a competitor's "fast" thermal cycler (5°C/sec). Actual run times are plotted. The time saved by using a protocol optimized for fast PCR is substantial (56–65 min), whereas the additional time saved by a faster-ramping cycler is relatively small (6–8 min).

As described above, cycling time can also be reduced by converting from a three-step to a two-step protocol in which the annealing and extension steps are combined at a temperature optimal for primer annealing yet sufficient for primer extension. Such two-step PCR protocols generate yields similar to three-step protocols for products up to 200 bp (Cha and Thilly 1995). Furthermore, a combined annealing and extension step at 60°C is typical for qPCR assays using TaqMan probes, and reaction efficiencies of around 100% are routinely achieved for such assays. This suggests that the processivity of *Taq* at this lower temperature is sufficient to fully extend products of 70–200 bp.

Final Extension

A post-PCR final incubation step of 5–10 min at 72°C is often recommended to promote complete synthesis of all PCR products. Although this is commonly referred to as an extension step, a major purpose is to allow reannealing of the PCR product into double-stranded DNA so it can be visualized using ethidium bromide after gel electrophoresis or used for cloning. We found that this step can be shortened to 30–60 sec for PCR products of 100–1,000 bp (Figure 5).

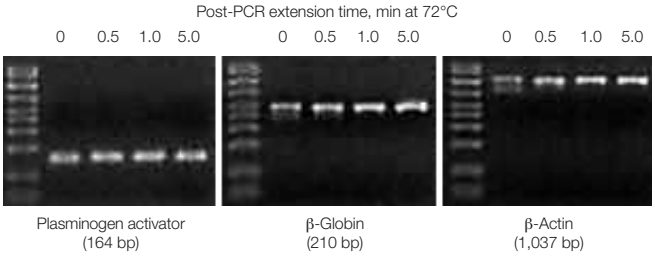

Fig. 5. The final extension step can be reduced to 1 min or less. Targets of 164–1,037 bp were amplified from human genomic DNA using a fast PCR protocol, then a final step of 0–5 min at 72°C was performed before gel analysis. Cycling protocol for 164 bp PCR product: 98°C, 30 sec; then 35 cycles of 92°C, 1 sec and 68°C, 15 sec. Actual run time, 33–38 min. Cycling protocol for 505 and 1,037 bp PCR products: 98°C, 30 sec; then 35 cycles of 92°C, 1 sec and 68°C, 30 sec. Actual run time, 41–46 min.

Number of Cycles

PCR can be completed in relatively few cycles (<20) if the starting target concentration is high. When starting with lower copy numbers (e.g., 100 copies) of target DNA, 35 cycles of PCR are generally adequate to detect the resulting product on a gel stained with ethidium bromide. With less starting target, additional cycles may be necessary. In practice, the amount of target is often unknown and may be only a few hundred copies per reaction. For this reason, researchers usually prefer to run 30–45 cycles of PCR despite the potential time savings of running fewer cycles.

Fast Real-Time qPCR

Our guidelines for fast PCR can be applied to other PCR applications, including real-time qPCR. For qPCR, primers are usually designed to amplify relatively short targets (70–200 bp) to ensure maximum efficiency. Such short targets may not require long denaturation and extension times, making them particularly suitable for modification for fast PCR assays without the need for special reagents, plastics, or instrumentation.

SYBR Green I Chemistry

Bio-Rad has traditionally recommended using a two-step, rather than a three-step, protocol for any real-time qPCR using SYBR Green chemistry. Run times can be further shortened by minimizing hold times using the same considerations used for conventional PCR. Figure 6 shows data from a modified real-time qPCR assay of a lambda DNA PCR product using iQ™ SYBR® Green supermix. In this assay, primer concentrations were increased to 0.75 µM. As this figure illustrates, it is possible to obtain reproducible results, minimal variance around the standard curve, and reaction efficiencies close to 100% for real-time PCRs completed in under 40 min.

For SYBR Green assays, we recommend running a post-amplification melt-curve analysis. This will lengthen the overall run time by approximately 10 min, but will provide valuable data on reaction specificity. The presence of a single product in the melt-curve analysis (Figure 6C) indicates the high specificity of the reaction.

Dual-Labeled Probes

Quantitative PCR using dual-labeled probes (often called TaqMan or 5' nuclease assays) uses a two-step PCR protocol with a combined annealing and extension step, commonly performed at 60°C. A combined annealing and extension step is necessary because the fluorescent chemistry requires the probe to be annealed to its target while the product is being extended. Again, significant run time reductions can be made simply by reducing hold times at each step. Table 1 shows data from a TaqMan real-time qPCR run under three different thermal cycling conditions. These real-time PCRs used iTaq supermix with ROX and were performed on the iQ™5 system, which has a maximum ramp rate of 3.3°C/sec. The unmodified protocol used manufacturer-recommended reaction conditions and yielded a run time of 68 min. Run time was reduced to 61 min when the reaction was run using a competitor's fast PCR protocol, which is designed for use with their reagents

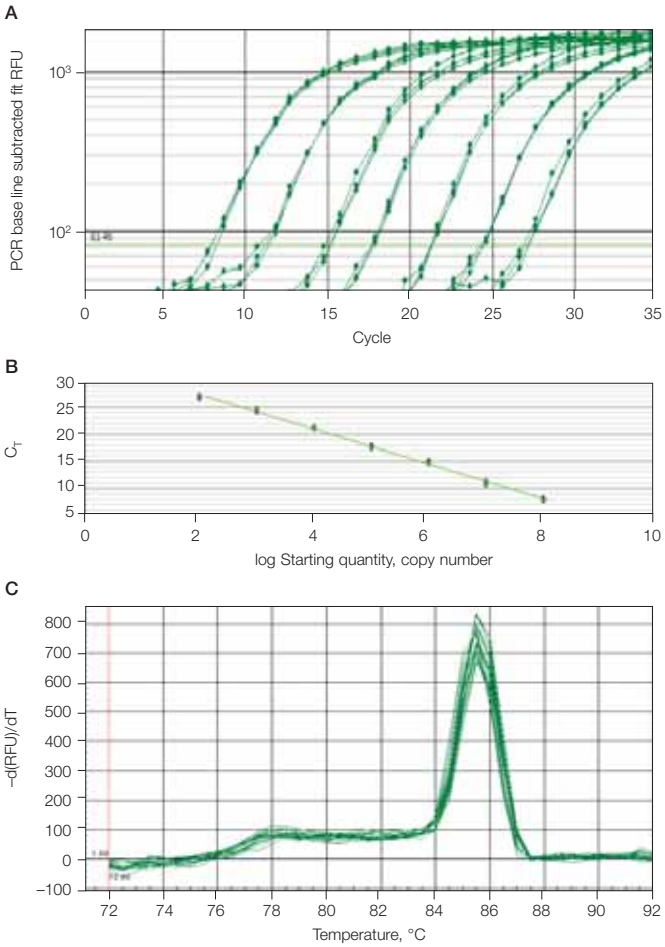

Fig. 6. SYBR Green real-time qPCR completed in under 40 min. Fluorescence curves (A), standard curve (B), and melt curves (C) are shown for a 150 bp region amplified from lambda DNA. Standard curve had y = –3.209x + 33.380, R^2 = 0.998; PCR efficiency = 104.9%. Cycling protocol was 98°C, 30 sec; then 35 cycles of 92°C, 1 sec and 70°C, 15 sec; then melt curve. Actual qPCR time, 37 min, 38 sec. Total run time with melt curve, 47 min, 36 sec.

and instruments specialized for fast PCR. Using a faster protocol achieved a further reduction of 15 min, resulting in an overall run time of 46 min. This protocol used a 30 sec initial denaturation and enzyme activation step at 98°C, and reduced hold times during cycling — 1 sec at 92°C for denaturation and 15 sec for annealing and extension. Each of these runs produced virtually identical results, with the maximum difference in average C_T between runs being 0.5 or less.

Table 1. Real-time qPCR with dual-labeled probes completed in under an hour. Results are shown for real-time PCR assays using dual-labeled probes. Identical reactions amplifying β-actin from human liver cDNA using iTaq supermix with ROX were run on the iQ5 real-time system using different thermal cycling conditions.

	Unmodified Protocol	Competitor's Fast Protocol	Modified Faster Protocol
Protocol	95°C, 3 min; then 40 cycles of 92°C, 10 sec and 60°C, 30 sec	95°C, 20 sec; then 40 cycles of 92°C, 3 sec and 60°C, 30 sec	98°C, 30 sec; then 40 cycles of 92°C, 1 sec and 60°C, 15 sec
Actual run time	68 min	61 min	46 min
Standard curve equation	y = –3.445x + 24.647	y = –3.376x + 24.895	y = –3.396x + 24.880
R^2 value	1.000	0.998	0.999
Reaction efficiency	95.1%	97.8%	97.0%
C_T values*			
Dilution factor			
10^{-5}	35.08 ± 0.107	34.82 ± 0.475	34.88 ± 0.267
10^{-4}	31.52 ± 0.097	31.83 ± 0.282	31.67 ± 0.132
10^{-3}	27.92 ± 0.031	28.34 ± 0.107	28.42 ± 0.272
10^{-2}	24.71 ± 0.054	24.89 ± 0.066	25.02 ± 0.175
10^{-1}	21.27 ± 0.066	21.58 ± 0.049	21.50 ± 0.146
1	17.71 ± 0.113	18.03 ± 0.022	17.93 ± 0.127

* Mean C_T ± SD for 3 or more replicates.

Saving Time in Long PCR

In general, longer targets (above 1 kb) need longer extension times, resulting in runs that can last several hours. The extremely high processivity of iProof polymerase enables extension to be completed in much less time and with less enzyme than is required for other polymerases. Elevated annealing temperatures (T_m + 3°C for oligonucleotides >20 bp) are recommended for iProof polymerase due to the higher salt concentration in the reaction buffer, so two-step protocols are routinely performed with this enzyme. Figure 7 compares the results of long PCR (2–20 kb) using iTaq, iProof, and PfuUltra polymerases in a two-step protocol with the manufacturer's recommended conditions. Time savings of up to 3- to 4-fold, as well as increased reaction success, were obtained with iProof polymerase.

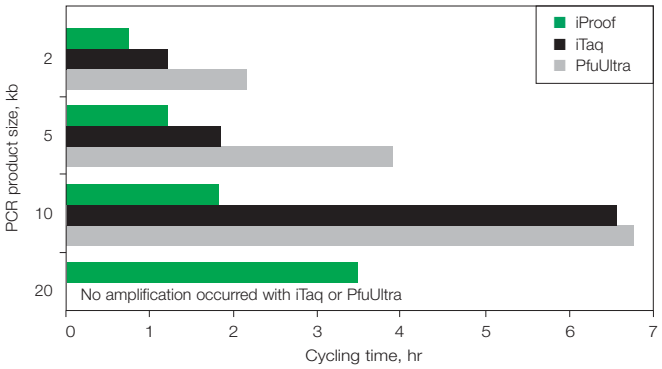

Fig. 7. Long PCR (up to 20 kb) can be achieved 3–4 times faster using iProof polymerase. Targets were amplified from lambda DNA using different polymerases. Primers were designed with T_m = 70–72°C, so that manufacturer-recommended protocols could be converted to two-step protocols. Protocols were run using a range of annealing/extension times; the fastest run time that produced a successful result (based on agarose gel electrophoresis) is plotted.

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Information in this tech note was current as of the date of writing (2006) and not necessarily the date this version (rev A, 2006) was published.

Addendum — Tips for Shortening Run Times

General Considerations for Fast PCR

To speed your reactions, consider altering your protocol, reaction mix, and PCR product size according to the following recommendations.

1. Protocol

- Begin with this fast PCR protocol template: 98°C, 30 sec; then 35 cycles of 92°C, 1 sec and 70°C, 15 sec; then 72°C, 1 min
- Modify the annealing/extension temperature so that it is halfway between 72°C and the average of the primer T_m values; for example, if the average primer T_m is 58°C, use an annealing/extension temperature of 65°C
- Alternatively, employ the rapid optimization strategy (below) that uses temperature gradients to optimize both speed and specificity
- If the starting target number might be <100 copies, perform 40 cycles

2. Reaction Mix

- For targets <1 kb, use an antibody-mediated hot-start polymerase such as iTaq; for targets >1 kb, use highly processive iProof polymerase
- If using existing primers, verify that T_m values are in the range of 58–72°C; if designing new primers, specify T_m values near 70°C*

3. PCR Product Size

Any size PCR product up to 20 kb can be amplified using these fast PCR guidelines. For fastest reactions, however, amplify targets <250 bp.

Rapid Optimization Strategy for Fast PCR

This simple strategy can quickly optimize a PCR reaction for minimal hold times, minimal ramping time, and shortest overall run times.

- Begin with this fast PCR protocol template: 98°C, 30 sec; then 35 cycles of 92°C, 1 sec, xx°C, 15 sec; then 72°C, 1 min, where xx = temperature gradient (see below)
- Use a temperature gradient (e.g., 0–10°C above the lowest primer T_m) to find the highest possible annealing/extension temperature
- Perform a second run with a temperature gradient (e.g., 85–95°C) to find the lowest possible denaturation temperature during cycling

Example:

Optimization Stage	PCR Protocol	Run Time
Before optimization	95°C, 3 min; then 35 cycles of 95°C, 15 sec, 60°C, 30 sec, 72°C, 30 sec; then 72°C, 10 min	88 min
Hold times reduced and annealing and extension steps combined using guidelines	98°C, 30 sec; then 35 cycles of 95°C, 1 sec, 60°C, 15 sec; then 72°C, 1 min	60 min
Gradient used to minimize temperature excursions	98°C, 30 sec; then 35 cycles of 90°C, 1 sec, 65°C, 15 sec; then 72°C, 1 min	36 min

Troubleshooting Fast PCR

If your fast PCR results in weak or nonspecific gel bands, adjust your reaction conditions in one of the following ways.

Symptom	Recommendation
Weak gel band	Increase the annealing/extension time in 5 sec increments Lower the annealing/extension temperature by 2 or 4°C Raise the denaturation temperature by 1 or 2°C
Nonspecific bands	Raise the annealing/extension temperature by 2 or 4°C Redesign primers to have 2–4°C higher T_m values, or to amplify a different region of the target sequence

* We recommend designing and verifying primer T_m values with a calculator such as Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Rozen and Skaletsky 2000), which references the thermodynamic parameters of Breslauer et al. (1986) and Rychlik et al. (1990). The various oligo T_m calculators available on web sites can give quite different T_m values for the same oligonucleotide.

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