October 2012

QIAGEN® Fast Cycling PCR Handbook

For fast and specific PCR on any thermal cycler

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

QIAGEN is the leading provider of innovative sample and assay technologies, enabling the isolation and detection of contents of any biological sample. Our advanced, high-quality products and services ensure success from sample to result.

QIAGEN sets standards in:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

Our mission is to enable you to achieve outstanding success and breakthroughs. For more information, visit www.qiagen.com .

Contents

Kit Contents

QIAGEN Fast Cycling PCR Kit	(50)	(200)	(1000)
Number of 20 µl reactions	**50**	**200**	**1000**
Catalog no.	**203741**	**203743**	**203745**
QIAGEN Fast Cycling PCR Master Mix, 2x	0.5 ml	2 x 1 ml	10 x 1 ml
CoralLoad® Dye, 10x	1 ml	1 ml	2 x 1 ml
Q-Solution®, 5x	2 ml	2 ml	2 x 2 ml
RNase-Free Water	1.9 ml	2 x 1.9 ml	6 x 1.9 ml
Quick-Start Protocol	1	1	1

Storage

The QIAGEN Fast Cycling PCR Kit is shipped on dry ice but retains full activity at room temperature (15–25°C) for 3 days. The QIAGEN Fast Cycling PCR Kit should be stored immediately upon receipt at –20°C in a constant temperature freezer. When stored under these conditions and handled correctly, this product can be kept at least until the expiration date (see the inside of the kit lid) without showing any reduction in performance.

Intended Use

The QIAGEN Fast Cycling PCR Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Quality Control

QIAGEN Fast Cycling PCR Master Mix:

Fast Cycling PCR assay performance:	Sensitivity, reproducibility, and specificity in fast-cycling PCR are tested in parallel reactions.

Buffers and reagents:

CoralLoad Dye, 10x:	Dye concentrations are tested.
Q-Solution, 5x:	Conductivity, pH, total aerobic microbial count, and performance in PCR are tested.
RNase-free water:	Conductivity, pH, and RNase activities are tested.

Product Specifications

QIAGEN Fast Cycling PCR Master Mix:	2x concentrated. Contains HotStarTaq *Plus* DNA Polymerase, Fast Cycling PCR Buffer, and dNTPs, optimized Mg^{2+} concentration

Buffers and reagents:

RNase-free water:	Ultrapure quality, PCR-grade
CoralLoad Dye:	10x concentrated. Contains orange dye and red dye
Q-Solution:	5x concentrated

Introduction

The QIAGEN Fast Cycling PCR Kit has been developed by QIAGEN to provide the unrivaled performance of HotStarTaq® *Plus* DNA Polymerase with a significantly shortened PCR cycling time on all PCR instruments. The combination of HotStarTaq *Plus* DNA Polymerase and the new QIAGEN Fast Cycling PCR Buffer in a convenient master mix format minimizes nonspecific amplification products, primer–dimers, and background while significantly reducing PCR duration (to as little as 20 minutes for many thermal cyclers). PCR products up to 3.5 kb are reproducibly obtained from complex starting templates. The pre-optimized 3-step PCR protocol prevents the need for redesign of primers for established assays. The optional addition of CoralLoad Dye to the master mix enables direct loading of the PCR products onto agarose gels without the need to add gel loading buffer, saving additional time and resources. The QIAGEN Fast Cycling PCR Kit makes highly specific hot-start PCR extremely fast and simple, with minimal optimization required.

HotStarTaq *Plus* DNA Polymerase

HotStarTaq *Plus* DNA Polymerase contained in the QIAGEN Fast Cycling PCR Master Mix is a modified form of the recombinant 94 kDa *Taq* DNA Polymerase from QIAGEN. HotStarTaq *Plus* DNA Polymerase is provided in an inactive state with no polymerase activity at ambient temperatures. This prevents the formation of misprimed products and primer–dimers at low temperatures. HotStarTaq *Plus* DNA Polymerase is activated by a brief 5-minute, 95°C incubation step, which can easily be incorporated into existing thermal cycling programs. HotStarTaq *Plus* DNA Polymerase provides the same unrivaled high PCR specificity as HotStarTaq Polymerase and often increases the yield of the specific PCR product. PCR setup is quick and convenient as all reaction components can be combined at room temperature.

QIAGEN Fast Cycling PCR Buffer

The new QIAGEN Fast Cycling PCR Buffer facilitates amplification of specific PCR products with significantly reduced cycling time. Based on the original QIAGEN PCR Buffer, this new formulation enables a high ratio of specific-to-nonspecific primer binding during the short annealing step of every PCR cycle. Owing to a uniquely balanced combination of KCl and $(NH_4)_2SO_4$, the PCR buffer provides stringent primer-annealing conditions over a wider range of annealing temperatures and Mg^{2+} concentrations than conventional PCR buffers. Optimization of PCR by varying the annealing temperature or the Mg^{2+} concentration is dramatically reduced and often not required. The patent–pending Fast Cycling PCR Buffer contains the novel Q-Bond® Molecule, which increases the affinity of *Taq* DNA polymerases for short single-stranded DNA fragments, reducing the time required for successful primer annealing to just 5 seconds. Denaturation and extension times are also reduced due to the unique buffer composition, which supports the melting behavior of DNA.

CoralLoad Dye

The QIAGEN Fast Cycling PCR Kit is supplied with new CoralLoad Dye that can be used to directly load the PCR onto an agarose gel without the need to add a gel loading buffer. CoralLoad Dye contains two marker dyes — an orange dye and a red dye — that facilitate estimation of DNA migration distance and optimization of agarose gel run time. CoralLoad Dye can be added to the PCR without affecting amplification sensitivity or specificity. PCR fragments amplified in the presence of CoralLoad Dye have been successfully tested for cloning and restriction digestion without prior purification.

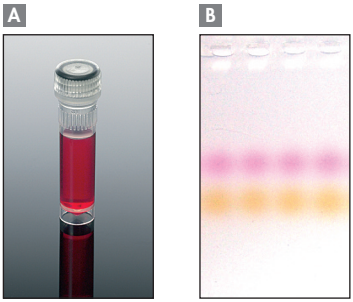


Figure 1. Ready-to-load PCR products. CoralLoad Dye A contains gel-tracking dyes B enabling immediate gel loading of PCR and easy visualization of DNA migration.

Q-Solution

QIAGEN Fast Cycling PCR Kit is also supplied with Q-Solution, an innovative PCR additive that facilitates amplification of difficult templates by modifying the melting behavior of DNA. This unique reagent will often enable or improve a suboptimal PCR caused by templates that have a high degree of secondary structure or that are GC-rich. Unlike other commonly used PCR additives such as DMSO, Q-Solution is used at just one working concentration, it is nontoxic, and PCR purity is guaranteed. For further information, please read the protocol "PCR Using the QIAGEN Fast Cycling PCR Master Mix and Q-Solution", page 16.

Specificity and Sensitivity

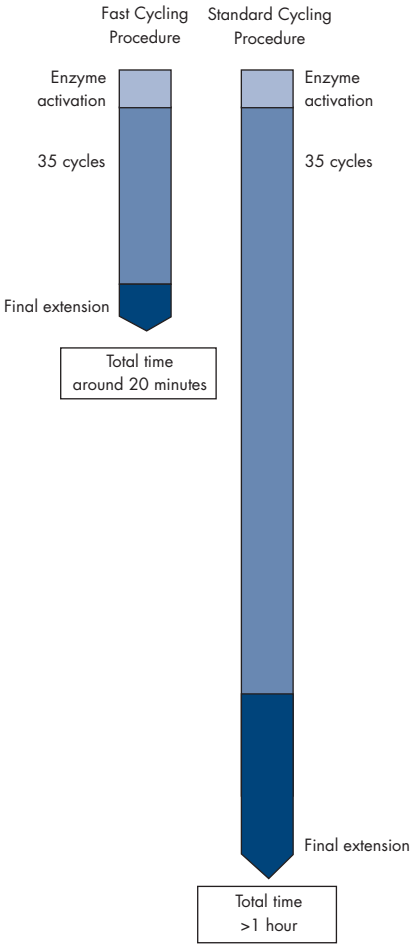
HotStarTaq *Plus* DNA Polymerase in combination with the new QIAGEN Fast Cycling PCR Buffer with its balanced potassium and sodium salts promotes specific primer–template annealing and simultaneously reduces nonspecific annealing. Maximum yields of specific products are obtained even when using extremely low template amounts (Appendix D, page 29).

Time and cost savings

The QIAGEN Fast Cycling PCR Kit allows increased PCR throughput using existing lab equipment. PCR cycling times can be reduced from around 1.5 hours to as little as 20 minutes, giving a total PCR time of just 30 minutes (Figure 2).* This time saving of up to 75% allows more time to be spent on downstream analysis. In addition, the bottlenecks caused by insufficient numbers of thermal cyclers and the need to purchase additional equipment can be eliminated.

* 30-minute PCR times were achieved using the latest fast-ramping thermal cyclers.

A



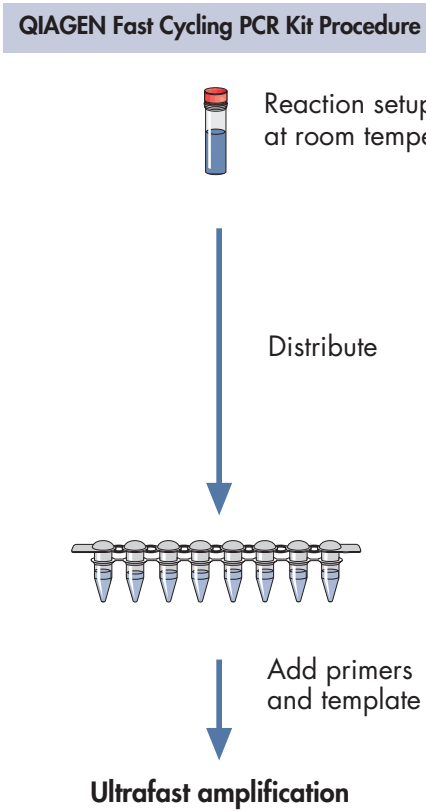
B

Fragment length	QIAGEN Fast Cycling procedure (min)	Standard cycling procedure (min)	Time saving
200 bp	15	68	78%
500 bp	20	68	71%
1000 bp	29	85	66%
3000 bp	63	155	59%

Figure 2. Significant time savings. A The QIAGEN Fast Cycling PCR Kit drastically decreases the time required for highly specific hot-start PCR. B Significant time savings can be made for all fragments <4 kb in length. 20 minute cycling time was achieved using a 500 bp PCR product (see table). Times stated do not include ramping times.

Principle and procedure

The master mix format, room temperature setup, and optional CoralLoad Dye result in a highly streamlined and convenient procedure (see flowchart).



Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Reaction tubes
- Pipets and pipet tips (aerosol resistant)
- Thermal cycler
- Vortexer
- Primers
  - Primers should be purchased from an established oligonucleotide manufacturer, such as Operon Biotechnologies (www.operon.com). Lyophilized primers should be dissolved in TE buffer to provide a stock solution of 100 µM; concentration should be checked by spectrophotometry. Primer stock solutions should be stored in aliquots at –20°C.

Protocol: PCR using the QIAGEN Fast Cycling PCR Kit

Important points before starting

- QIAGEN Fast Cycling PCR Master Mix requires a hot-start PCR activation step of **5 min at 95°C** (see step 5 of this protocol).
- For optimal results a temperature of **96°C** is strongly recommended for the **denaturation step**. This temperature compensates for variability in temperature and time calculation between different thermal cyclers.
- A default primer concentration of **0.5 µM** (each primer) is recommended.
- When using the **Eppendorf® Mastercycler® ep S** in block mode, use a **denaturation step of 5 s at 98°C** due to the extremely fast ramp times achieved with this instrument.
- If performing PCR in **384-well PCR plates**, we strongly advise the use of PCR plates recommended by the thermal cycler manufacturer to ensure perfect fit of plates for optimal heat transfer.
- **Annealing temperatures >60°C** are not generally recommended. When using **fluorescently labeled primers**, annealing temperature may need to be lowered compared with unmodified primers.
- **CoralLoad Dye** is not recommended when downstream applications require fluorescence or absorbance measurements without an intermediate purification of the PCR product (e.g., using QIAquick® PCR Purification Kits or MinElute® PCR Purification Kits).

Procedure

1. **Thaw QIAGEN Fast Cycling PCR Master Mix, primer solutions, nucleic acid template, RNase-free water, and optionally 10x CoralLoad Dye.**

   Mix well before use.

2. **Briefly vortex and dispense 10 µl QIAGEN Fast Cycling PCR Master Mix (and optionally 2 µl 10x CoralLoad Dye) into each PCR tube according to Table 1.**

   It is important to mix the QIAGEN Fast Cycling PCR Master Mix before use to avoid localized concentrations of salt.

   It is not necessary to keep reaction vessels on ice since HotStarTaq *Plus* DNA Polymerase is inactive at room temperature.

Table 1. Reaction composition using QIAGEN Fast Cycling PCR Master Mix

Component	Volume/reaction	Final concentration
QIAGEN Fast Cycling PCR Master Mix	10 µl	Contains HotStarTaq *Plus* DNA Polymerase, 1x Fast Cycling Buffer, 200 µM of each dNTP, optimized Mg^{2+} concentration
Optional: 10x CoralLoad Dye	2 µl	1x
Diluted primer mix		
Primer A	Variable	0.5 µM
Primer B	Variable	0.5 µM
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	<300 ng/20 µl reaction
Total volume	**20 µl***	–

* Do not use reaction volumes larger than 20 µl as this will interfere with the optimal temperature gradient required for successful results.

3. **Distribute the appropriate volume of diluted primer mix into the PCR tubes containing the master mix.**
4. **Add template DNA (<300 ng/20 µl reaction) to the individual PCR tubes.**

   For RT-PCR, add an aliquot from the reverse transcriptase reaction. The volume added should not exceed 10% of the final PCR volume (see Appendix E, page 30).
5. **Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 2.**

   Note: Each PCR program must start with an initial heat activation step at 95°C for 5 min.

   A typical PCR cycling program is outlined in Table 2. For maximum yield and specificity, temperatures and cycling times may be optimized for each new template target or primer pair.

   Note: PCR duration is determined by the length of the PCR product being amplified. When amplifying multiple PCR products of differing lengths, always choose protocol parameters for the longest PCR product.

Table 2. Optimized cycling protocol

			Additional comments
Initial activation step:	5 min	95°C	HotStarTaq *Plus* DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation:	5 s	**96°C**	Note: If using an **Eppendorf Mastercycler ep S** in block mode the denaturation should be performed at 98°C.
Annealing:	5 s	60°C*	An annealing temperature >60°C is not recommended. Use a temperature approximately 5°C below T_m of primers (see Appendix B, page 27).*
Extension:	3 s/ 100 bp	**68°C**	Use an extension time of 3 s per 100 bp DNA (i.e., 15 s for 500 bp fragments, 30 s for 1 kb fragments).†
Number of cycles:	30–40		See Appendix C, page 29.
Final extension:	1 min	72°C	

* For existing primer–template pairs, use the previously defined optimized annealing temperature. However, an annealing temperature of 60°C works for most primer-template pairs and should generally not be exceeded.

† This extension rate provides a maximal fragment length of 3.5 kb when amplifying complex templates such as genomic DNA. Longer fragment lengths are possible if using plasmid DNA.

6. **Place the PCR tubes in the thermal cycler and start the cycling program.**

   Note: After amplification, samples can be stored overnight at 2–8°C or at –20°C for longer storage.

7. **When using CoralLoad Dye, the PCR can be directly loaded onto an agarose gel without prior addition of a PCR loading buffer and gel tracking dyes.**

   CoralLoad Dye contains gel tracking dyes. Please refer to Table 3 to identify the dyes according to migration distance and agarose gel percentage and type.

   Note: Due to the high viscosity of the solution, apply the solution slowly into the wells of the agarose gel.

Table 3. Migration distance of gel tracking dyes

%TAE (TBE) agarose gel	Red dye	Orange dye
0.8	500 bp (270 bp)	~80 bp (<10 bp)
1.0	300 bp (220 bp)	~40 bp (<10 bp)
1.5	250 bp (120 bp)	~20 bp (<10 bp)
2.0	100 bp (110 bp)	<10 bp (<10 bp)
3.0	50 bp (100 bp)	<10 bp (<10 bp)

Protocol: PCR using the QIAGEN Fast Cycling PCR Kit and Q-Solution

This protocol is designed for using Q-Solution in PCR assays. Q-Solution changes the melting behavior of DNA and can be used for PCR systems that do not work well under standard conditions. When using Q-Solution the first time for a particular primer–template pair, always perform parallel reactions with and without Q-Solution. This recommendation should also be followed if another PCR additive (such as DMSO) was previously used for a particular primer–template pair.

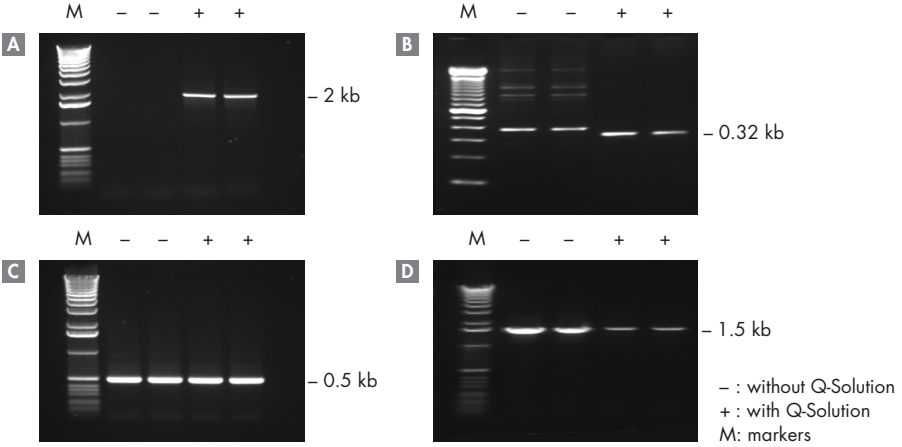
When using Q-Solution, the following effects may be observed depending on the individual PCR assay:

Case A: Q-Solution enables amplification of a reaction that previously failed.

Case B: Q-Solution increases PCR specificity in certain primer–template systems.

Case C: Q-Solution has no effect on PCR performance.

Case D: Q-Solution causes reduced efficiency or failure of a previously successful amplification reaction. In this case, addition of Q-Solution disturbs the previously optimal primer–template annealing. Therefore, when using Q-Solution for the first time for a particular primer–template system, always perform reactions in parallel with and without Q-Solution.



Important points before starting

- HotStarTaq *Plus* DNA Polymerase requires an activation step of **5 min at 95°C** (see step 5 of this protocol).
- For optimal results a temperature of **96°C** is strongly recommended for the **denaturation step**. This temperature compensates for variability in temperature and time calculation between different thermal cyclers.

- A default primer concentration of **0.5 µM** (each primer) is recommended.
- When using the **Eppendorf Mastercycler ep S** in block mode, use a **denaturation step of 5 s at 98°C** due to the extremely fast ramp times achieved with this instrument.
- If performing PCR in **384-well PCR plates**, we strongly advise the use of PCR plates recommended by the thermal cycler manufacturer to ensure perfect fit of plates for optimal heat transfer.
- **Annealing temperatures >60°C** are not generally recommended. When using **fluorescently labeled primers**, annealing temperature may need to be lowered compared with unmodified primers.
- **CoralLoad Dye** is not recommended when downstream applications require fluorescence or absorbance measurements without an intermediate purification of the PCR product (e.g., using QIAquick PCR Purification Kits or MinElute PCR Purification Kits).
- When using **Q-Solution** it is important to perform parallel amplification reactions without Q-Solution.

Procedure

1. **Thaw QIAGEN Fast Cycling PCR Master Mix, primer solutions, nucleic acid template, 5x Q-Solution, RNase-free water, and optionally 10x CoralLoad Dye.**

   Mix well before use.

2. **Briefly vortex and dispense 10 µl QIAGEN Fast Cycling PCR Master Mix, 4 µl Q-Solution (and optionally 2 µl 10x CoralLoad Dye) into each PCR tube according to Table 4.**

   It is important to mix the QIAGEN Fast Cycling PCR Master Mix before use to avoid localized concentrations of salt.

   It is not necessary to keep reaction vessels on ice since HotStarTaq *Plus* DNA Polymerase is inactive at room temperature.

3. **Distribute the appropriate volume of diluted primer mix into the PCR tubes containing the master mix.**

4. **Add template DNA (<300 ng/20 µl reaction) to the individual PCR tubes.**

   For RT-PCR, add an aliquot from the reverse transcriptase reaction. The volume added should not exceed 10% of the final PCR volume (see Appendix E, page 30).

Table 4. Reaction composition using QIAGEN Fast Cycling PCR Master Mix

Component	Volume/reaction	Final Concentration
QIAGEN Fast Cycling PCR Master Mix	10 µl	Contains HotStarTaq *Plus* DNA Polymerase, 1x Fast Cycling Buffer, 200 µM of each dNTP, optimized Mg^{2+} concentration
5x Q-Solution	4 µl	1x
Optional: 10x CoralLoad Dye	2 µl	1x
Diluted primer mix		
Primer A	Variable	0.5 µM
Primer B	Variable	0.5 µM
RNase-Free Water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	<300 ng/20 µl reaction
Total volume	**20 µl***	–

* Do not use reaction volumes larger than 20 µl as this will interfere with the optimal temperature gradient required for successful results.

5. Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 5.

Note: Each PCR program must start with an initial heat activation step at 95°C for 5 min.

A typical PCR cycling program is outlined in Table 5. For maximum yield and specificity, temperatures and cycling times may be optimized for each new template target or primer pair.

Note: PCR duration is determined by the length of the PCR product being amplified. When amplifying multiple PCR products of differing lengths, always choose protocol parameters for the longest PCR product.

Table 5. Optimized cycling protocol

			Additional comments
Initial activation step:	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation:	5 s	96°C	Note: If using an **Eppendorf Mastercycler ep S** in block mode the denaturation should be performed at 98°C.
Annealing:	5 s	60°C*	An annealing temperature >60°C is not recommended. Use a temperature approximately 5°C below T_m of primers (see Appendix B, page 27).*
Extension:	3s/ 100 bp	**68°C**	Use an extension time of 3 s per 100 bp DNA (i.e., 15 s for 500 bp fragments, 30 s for 1 kb fragments).†
Number of cycles:	30–40		See Appendix C, page 29.
Final extension:	1 min	72°C	

* For existing primer-template pairs, use the previously defined optimized annealing temperature. However, an annealing temperature of 60°C works for most primer-template pairs and should generally not be exceeded.

† This extension rate provides a maximal fragment length of 3.5 kb when amplifying complex templates such as genomic DNA. Longer fragment lengths are possible if using plasmid DNA.

6. **Place the PCR tubes in the thermal cycler and start the cycling program.**

   Note: After amplification, samples can be stored overnight at 2–8°C or at –20°C for longer storage.

7. **When using CoralLoad Dye, the PCR can be directly loaded onto an agarose gel without prior addition of a PCR loading buffer and gel tracking dyes.**

   CoralLoad Dye contains gel tracking dyes. Please refer to Table 6 to identify the dyes according to migration distance and agarose gel percentage and type.

   Note: Due to the high viscosity of the solution, apply the solution slowly into the wells of the agarose gel.

   Note: CoralLoad Dye does not interfere with subsequent enzymatic reactions such as cloning or restriction analysis.

Table 6. Migration distance of gel tracking dyes

%TAE (TBE) agarose gel	Red dye	Orange dye
0.8	500 bp (270 bp)	~80 bp (<10 bp)
1.0	300 bp (220 bp)	~40 bp (<10 bp)
1.5	250 bp (120 bp)	~20 bp (<10 bp)
2.0	100 bp (110 bp)	<10 bp (<10 bp)
3.0	50 bp (100 bp)	<10 bp (<10 bp)

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

		Comments and suggestions
Little or no product		
a)	HotStarTaq *Plus* DNA Polymerase not activated	Check whether PCR was started with an initial incubation step at 95°C for 5 min.
b)	Incorrect denaturation temperature or time	Always use a 5 s denaturation step at 96°C. If using the Eppendorf Mastercycler ep S in block mode, use a 5 s denaturation step at 98°C.
c)	Primer concentration not optimal or primers degraded	Use 0.5 µM of each primer. In particular, when performing highly sensitive PCR, check for possible degradation of the primers on a denaturing polyacrylamide gel.*
d)	PCR tubes or plates do not fit cycler block perfectly	Use PCR tubes or plates recommended by the thermal cycler manufacturer. This is of particular importance when performing PCR in **384-well PCR plates**.
e)	Modified primers used	When using modified primers such as fluorescence-labeled primers, lower the annealing temperature in 2°C steps or perform a temperature gradient PCR.
f)	Pipetting error or missing reagent	Repeat the PCR. Check the concentrations and storage conditions of reagents, including primers. Ensure a 1:1 ratio of Fast Cycling PCR Master Mix to primer-template solution is maintained.
g)	PCR cycling conditions are not optimal	Using the same cycling conditions, repeat the PCR using Q-Solution. Follow the protocol on page 16.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

		Comments and suggestions
h)	Insufficient number of cycles	Increase the number of cycles in steps of 5 cycles (see Appendix C, page 29).
i)	Problems with starting template	Check the concentration, storage conditions, and quality of the starting template (see Appendix A, page 25). If necessary, make new serial dilutions of template nucleic acid from stock solutions. Repeat the PCR using the new dilutions.
j)	Incorrect annealing temperature or time	Decrease annealing temperature in 2°C steps. Annealing time should be 5 s. Difficulties in determining the optimal annealing temperature can be overcome in many cases by performing a temperature gradient PCR if the thermal cycler has a temperature gradient function.
k)	Extension time too short	Allow an extension time of 3 s per 100 bp amplified DNA. If amplifying PCR products of differing lengths, choose an extension time based on the longest expected product.
l)	Primer design not optimal	Review primer design (see Appendix B, page 27).
m)	RT reaction error	For RT-PCR, take into consideration the efficiency of the reverse transcriptase reaction, which averages 10–30%. The added volume of reverse transcriptase reaction should not exceed 10% of the final PCR volume (see Appendix E, page 30).
n)	PCR of long fragments from genomic DNA	When amplifying products longer than 4 kb from genomic DNA, increase the concentration of genomic DNA in the reaction (see Appendix A, page 25).
o)	PCR overlaid with mineral oil when using a thermal cycler with a heated lid	When performing PCR in a thermal cycler with a heated lid, do not overlay the PCR samples with mineral oil if the heated lid is switched on as this may decrease the yield of PCR product.
p)	Problems with the thermal cycler	Check the power to the thermal cycler and that the thermal cycler has been correctly programmed.

		Comments and suggestions
Product is multi-banded		
a)	HotStarTaq *Plus* DNA Polymerase activation time too long	Check whether PCR was started with an initial incubation step at 95°C for only 5 min.
b)	PCR cycling conditions not optimal	Using the same cycling conditions, repeat the PCR using Q-Solution. Follow the protocol on page 16.
c)	Annealing temperature too low	Decrease annealing temperature in 2°C steps. Annealing time should be 5 s. Difficulties in determining the optimal annealing temperature can be overcome in many cases by performing a temperature gradient PCR if the thermal cycler has a temperature gradient function.
d)	Primer concentration not optimal or primers degraded	Default primer concentration is 0.5 µM of each primer. Repeat the PCR with different primer concentrations from 0.1–0.5 µM of each primer in 0.1 µM steps. In particular, when performing highly sensitive PCR, check for possible degradation of the primers on a denaturing polyacrylamide gel.*
e)	Primer design not optimal	Review primer design (see Appendix B, page 27).
Product is smeared		
a)	HotStarTaq *Plus* DNA Polymerase activation time too long	Check whether PCR was started with an initial incubation step at 95°C for only 5 min.
b)	Too much starting template	Check the concentration and storage conditions of the starting template (see Appendix A, page 25). Make serial dilutions of template nucleic acid from stock solutions. Perform PCR using these serial dilutions.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

		Comments and suggestions
c)	Carryover contamination	If the negative-control PCR (without template DNA) shows a PCR product or a smear, exchange all reagents. Use disposable pipet tips containing hydrophobic filters to minimize cross-contamination. Set up all reaction mixtures in an area separate from those used for DNA preparation or PCR product analysis.
d)	Too many cycles	Reduce the number of cycles in steps of 3 cycles.
e)	Primer concentration not optimal or primers degraded	Repeat the PCR with different primer concentrations from 0.1–0.5 µM of each primer (in 0.1 µM steps). In particular, when performing highly sensitive PCR, check for possible degradation of the primers on a denaturing polyacrylamide gel.*
f)	Primer design not optimal	Review primer design (see Appendix B, page 27).

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Appendix A: Starting Template

Both the quality and quantity of nucleic acid starting template affect PCR, in particular the sensitivity and efficiency of amplification.*

Quality of starting template

Since PCR consists of multiple rounds of enzymatic reactions, it is more sensitive to impurities such as proteins, phenol/chloroform, salts, ethanol, EDTA, and other chemical solvents than single-step enzyme-catalyzed processes. QIAGEN offers a complete range of nucleic acid preparation systems providing the highest-quality templates for PCR, such as the QIAprep® system for rapid plasmid purification, the QIAamp® and DNeasy® systems for rapid purification of genomic DNA and viral nucleic acids, and the RNeasy® system for RNA preparation from a variety of sources. For more information about these products, contact one of our Technical Service Departments (see back cover).

Quantity of starting template

The annealing efficiency of primer to template is an important factor in PCR. Owing to the thermodynamic nature of the reaction, the primer:template ratio strongly influences the specificity and efficiency of PCR and should be optimized empirically. If too little template is used, primers may not be able to find their complementary sequences. Too much template may lead to an increase in mispriming events. As an initial guide, spectrophotometric and molar conversion values for different nucleic acid templates are listed in Tables 7 and 8 respectively.

Table 7. Spectrophotometric conversions for nucleic acid templates

1 A_{260} unit†	Concentration (µg/ml)
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40

† Absorbance at 260 nm = 1

* For further information see our guide *Maximizing PCR and RT-PCR success — Second Edition*. To obtain a copy, visit the QIAGEN web site at www.qiagen.com or call one of the QIAGEN Technical Service Departments or local distributors (see back cover).

Table 8. Molar conversions for nucleic acid templates

Nucleic acid	Size	pmol/µg	Molecules/µg
1 kb DNA	1000 bp	1.52	9.1×10^{11}
pUC19 DNA	2686 bp	0.57	3.4×10^{11}
pTZ18R DNA	2870 bp	0.54	3.2×10^{11}
pBluescript® II DNA	2961 bp	0.52	3.1×10^{11}
Lambda DNA	48,502 bp	0.03	1.8×10^{10}
Average mRNA	1930 nt	1.67	1.0×10^{12}
Genomic DNA			
Escherichia coli	4.7×10^{6}*	3.0×10^{-4}	1.8×10^{8}†
Drosophila melanogaster	1.4×10^{8}*	1.1×10^{-5}	6.6×10^{5}†
Mus musculus (mouse)	2.7×10^{9}*	5.7×10^{-7}	3.4×10^{5}†
Homo sapiens (human)	3.3×10^{9}*	4.7×10^{-7}	2.8×10^{5}†

* Base pairs in haploid genome.

† For single-copy genes.

Appendix B: Primer Design, Concentration, and Storage

Standard PCR primers

Prerequisites for successful PCR include the design of optimal primer pairs, the use of appropriate primer concentrations, and the correct storage of primer solutions. Some general guidelines are given in Table 9.*

Table 9. General guidelines for standard PCR primers

Length:	18–30 nucleotides
G/C content:	40–60%
T_m :	Simplified formula for estimating melting temperature (T_m): T_m = 2°C x (A+T) + 4°C x (G+C) Whenever possible, design primer pairs with similar T_m values. Optimal annealing temperatures may be above or below the estimated T_m. As a starting point, use an annealing temperature 5°C below T_m.
Sequence:	■ Avoid complementarity of two or three bases at the 3' ends of primer pairs to reduce primer–dimer formation. ■ Avoid mismatches between the 3' end of the primer and the target-template sequence. ■ Avoid runs of 3 or more G or C at the 3' end. ■ Avoid a 3'-end T. Primers with a T at the 3' end have a greater tolerance of mismatch. ■ Avoid complementary sequences within a primer sequence and between the primer pair. ■ Commercially available computer software (e.g., Primer Designer 1.0, Scientific Software, 1990; Oligo, Rychlik, and Rhoads, 1989) can be used for primer design.
Concentration:	■ Spectrophotometric conversion for primers: 1 A_{260} unit = 20–30 µg/ml

Table continues on next page.

* For further information see our guide *Maximizing PCR and RT-PCR success — Second Edition*. To obtain a copy, visit the QIAGEN web site at www.qiagen.com or call one of the QIAGEN Technical Service Departments or local distributors (see back cover).

Table 9 (continued)

- Molar conversions:

Primer length	pmol/µg	20 pmol
18mer	168	119 ng
20mer	152	132 ng
25mer	121	165 ng
30mer	101	198 ng

- Use 0.1–0.5 µM of each primer in PCR. For most applications, a primer concentration of 0.2 µM will be sufficient.

Storage: Lyophilized primers should be dissolved in a small volume of distilled water or TE buffer* to make a concentrated stock solution. Prepare small aliquots of working solutions containing 10 pmol/µl to avoid repeated thawing and freezing. Store all primer solutions at –20°C. Primer quality can be checked on a denaturing polyacrylamide gel;* a single band should be seen.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Appendix C: Number of PCR Cycles

A cycling program usually consists of between 30 and 40 cycles, depending on the number of copies of the starting template. Too many cycles do not necessarily lead to a higher yield of PCR product; instead they may increase nonspecific background and decrease the yield of specific PCR product. Table 10 provides a general guideline for choosing the number of cycles.

Table 10. General guidelines for choosing the number of PCR cycles

Number of copies of starting template*	1 kb DNA	*E. coli* DNA†	Human genomic DNA†	Number of cycles
10–100	0.01–0.11 fg	0.05–0.56 pg	36–360 pg	40–45
100–1000	0.11–1.1 fg	0.56–5.56 pg	0.36–3.6 ng	35–40
1 x 10^3 – 5 x 10^4	1.1–55 fg	5.56–278 pg	3.6–179 ng	30–35
>5 x 10^4	>55 fg	>278 pg	>179 ng	25–35

* Refer to Table 8 (page 26) to calculate the number of molecules. When starting with cDNA templates, it is important to take into account the efficiency of reverse transcription in cDNA synthesis, which is on average 10–30%.

† Refers to single-copy genes.

Appendix D: Sensitive PCR Assays

PCR can be performed to amplify and detect just a single copy of a nucleic acid sequence. However, amplification of such a low number of target sequences is often limited by the generation of nonspecific PCR products and primer–dimers. The combination of HotStarTaq *Plus* DNA Polymerase and QIAGEN Fast Cycling PCR Buffer in the QIAGEN Fast Cycling PCR Kit increases specificity both at the start of and during PCR. Thus HotStarTaq *Plus* DNA Polymerase is well suited to such highly sensitive PCR assays.

Appendix E: RT-PCR

To perform PCR using RNA as a starting template, the RNA must first be reverse transcribed into cDNA in a reverse transcription reaction (RT reaction). Failure of the subsequent PCR is often a result of the limitations of the RT reaction. On average, only 10–30% of the original RNA molecules are reverse transcribed into cDNA. The expression level of the target RNA molecules and the relatively low efficiency of the reverse transcription reaction must be considered when calculating the appropriate amount of starting template for subsequent PCR. The volume of the RT reaction transferred should not exceed 10% of the total PCR volume. General guidelines are presented in Table 11.

Table 11. General guidelines for performing RT-PCR

RNA purification and reverse transcription:	QIAGEN offers the RNeasy system for total RNA isolation, Oligotex® Kits for messenger RNA isolation, and Omniscript® Reverse Transcriptase for reverse transcription.* Follow the detailed protocol in the *Omniscript Reverse Transcriptase Handbook*. Or, when using an enzyme from another supplier, follow the manufacturer's instructions. The following guidelines may be helpful. ■ Mix the following reagents in a microcentrifuge tube: 4.0 µl 5x RT buffer 1.0 µl RNase inhibitor (5 units/µl) 2.0 µl DTT (0.1 M) 1.0 µl each dNTP (10 mM) ~1 µg RNA 2.5 µl primer (0.2 µg/µl) Reverse transcriptase† Add RNase-free water to a final volume of 20 µl. ■ Incubate following the manufacturer's instructions. ■ Heat the reaction mix to 95°C for 5 min to inactivate the reverse transcriptase.
PCR:	■ Prepare a PCR mixture following steps 1–3 in protocols. ■ Add 2–5 µl from the RT reaction to each PCR tube containing the master mix. ■ Continue with step 5 in the PCR protocols.

Oligotex resin is not available in Japan.

* For further information about RNeasy, Oligotex, and Omniscript products, contact your local QIAGEN Technical Services or distributor (see back cover).

† Please refer to the manufacturer's instructions for the amount of enzyme required.

Appendix F: Purification of PCR Products

After amplification, the PCR sample contains a complex mixture of specific PCR product and residual reaction components such as primers, unincorporated nucleotides, enzyme(s), salts, mineral oil, and probably nonspecific amplification products. Before the specific PCR product can be used in subsequent experiments it is often necessary to remove these contaminants. The QIAquick system offers a quick and easy method for purifying the final PCR product. Using the MinElute system, PCR products can be purified in higher concentrations due to the low elution volumes needed in this system. Gel loading reagent and tracking dyes are effectively removed with the QIAquick and MinElute system. For more information about QIAquick and MinElute products, please call QIAGEN Technical Services or your local distributor (see back cover).

Appendix G: Control of Contamination

It is extremely important to include at least one negative control that lacks the template nucleic acid in every PCR setup to detect possible contamination.

General physical precautions

- Separate the working areas for setting up the PCR master mix and DNA handling, including the addition of starting template, PCR product analysis, or plasmid preparation. Ideally, use separate rooms.
- Use a separate set of pipets for the PCR master mix. Use of pipet tips with hydrophobic filters is strongly recommended.
- In case of contamination, laboratory benches, apparatus, and pipets can be decontaminated by cleaning them with a 1/10 dilution of a commercial bleach solution.*† Afterwards, the benches and pipets should be rinsed with distilled water.

General chemical precautions

- PCR stock solutions can also be decontaminated using UV light. This method is laborious, however, and its efficiency is difficult to control and cannot be guaranteed. We recommend storing solutions in small aliquots and using fresh aliquots for each PCR.
- Another approach to preventing amplification of contaminating DNA is to treat individual reaction mixtures with DNase I† or restriction enzymes† that cut between the binding sites of the amplification primers used, before adding the template DNA sample.

* Most commercial bleach solutions are approximately 5.25% sodium hypochlorate.

† When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc. For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Ordering Information

Product	Contents	Cat. no.
QIAGEN Fast Cycling PCR Kit (50)	For 50 x 20 µl reactions: 0.5 ml QIAGEN Fast Cycling PCR Master Mix, 10x CoralLoad Dye, Q-Solution, RNase-Free Water	203741
QIAGEN Fast Cycling PCR Kit (200)	For 200 x 20 µl reactions: 2 x 1 ml QIAGEN Fast Cycling PCR Master Mix, 10x CoralLoad Dye, 5x Q-Solution, RNase-Free Water	203743
QIAGEN Fast Cycling PCR Kit (1000)	For 1000 x 20 µl reactions: 10 x 1 ml QIAGEN Fast Cycling PCR Master Mix, 10x CoralLoad Dye, 5x Q-Solution, RNase-Free Water	203745
Related Products		
HotStarTaq DNA Polymerase (250 U)*	250 units HotStarTaq DNA Polymerase, Polymerase, 10x PCR Buffer (containing 15 mM $MgCl_2$), 5x Q-Solution, 25 mM $MgCl_2$	203203
HotStarTaq Master Mix (250 U)*	3 x 0.85 ml HotStarTaq Master Mix, containing 250 units HotStarTaq DNA Polymerase total and providing a final concentration of 1.5 mM $MgCl_2$ and 200 µM each dNTP; 2 x 1.7 ml distilled water	203443
HotStarTaq *Plus* DNA Polymerase (250 U)*	250 units HotStarTaq *Plus* DNA Polymerase, 10x PCR Buffer,† 10x CoralLoad PCR Buffer,† 5x Q-Solution, 25 mM $MgCl_2$	203603
HotStarTaq *Plus* Master Mix Kit (250)*	For 250 x 20 µl reactions: 3 x 0.85 ml HotStarTaq Plus Master Mix,* containing 250 units of HotStarTaq *Plus* DNA Polymerase total, 1 x 0.55 ml CoralLoad Concentrate, 2 x 1.9 ml RNase-Free Water	203643

* Larger kit sizes available, please inquire.

† Contains 15 mM $MgCl_2$.

Ordering Information

Product	Contents	Cat. no.
HotStar HiFidelity Polymerase Kit (100 U)*	100 units HotStar HiFidelity DNA Polymerase, 5x HotStar HiFidelity PCR Buffer (inc. dNTPs),† 5x Q-Solution, 25 mM $MgSO_4$, RNase-Free Water	202602
Taq DNA Polymerase (250 U)*	250 units *Taq* DNA Polymerase, 10x PCR Buffer,‡ 5x Q-Solution, 25 mM MgCl2	201203
TopTaq® DNA Polymerase (250)*	250 units TopTaq DNA Polymerase, 10x PCR Buffer,‡ 10x CoralLoad Concentrate, 5x Q-Solution, 25 mM $MgCl_2$	200203
QIAGEN Multiplex PCR Kit (100)*	For 100 x 50 µl multiplex PCR reactions: 2x QIAGEN Multiplex PCR Master Mix (providing a final concentration of 3 mM $MgCl_2$, 3 x 0.85 ml), 5x Q-Solution (1 x 2.0 ml), distilled water (2 x 1.7 ml)	206143
Omniscript RT Kit (10)*	For 10 reverse-transcription reactions: 40 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix (containing 5 mM each dNTP), RNase-free water	205110
Sensiscript® RT Kit (50)*	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix (containing 5 mM each dNTP), RNase-free water	205211
dNTP Mix, PCR Grade (200 µl)*	Mix containing 10 mM each of dATP, dCTP, dGTP, and dTTP (1 x 200 µl)	201900

* Larger kit sizes available, please inquire.

† Contains Factor SB, dNTPs, and optimized concentration of $MgSO_4$.

‡ Contains 15 mM $MgCl_2$.

Ordering Information

Product	Contents	Cat. no.
dNTP Set, PCR Grade (4 x 100 µl)*	100 mM each of dATP, dCTP, dGTP, and dTTP	201912
QIAquick PCR Purification Kit (50)*	50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28104
QIAquick Gel Extraction Kit (50)*	50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28704
DNeasy Blood and Tissue Kit (50)*	50 DNeasy Spin Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	69504
DNeasy Plant Mini Kit (50)*	50 DNeasy Mini Spin Columns, 50 QIAshredder Mini Spin Columns, RNase A, Buffers, Collection Tubes (2 ml)	69104
QIAamp DNA Mini Kit (50)*	For 50 DNA preps: 50 QIAamp Mini Spin Columns, Proteinase K, Reagents, Buffers, Collection Tubes (2 ml)	51304

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

* Larger kit sizes available, please inquire.

Notes

Notes

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Australia ▪ techservice-au@qiagen.com

Austria ▪ techservice-at@qiagen.com

Belgium ▪ techservice-bnl@qiagen.com

Brazil ▪ suportetecnico.brasil@qiagen.com

Canada ▪ techservice-ca@qiagen.com

China ▪ techservice-cn@qiagen.com

Denmark ▪ techservice-nordic@qiagen.com

Finland ▪ techservice-nordic@qiagen.com

France ▪ techservice-fr@qiagen.com

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Ireland ▪ techservice-uk@qiagen.com

Italy ▪ techservice-it@qiagen.com

Japan ▪ techservice-jp@qiagen.com

Korea (South) ▪ techservice-kr@qiagen.com

Luxembourg ▪ techservice-bnl@qiagen.com

Mexico ▪ techservice-mx@qiagen.com

The Netherlands ▪ techservice-bnl@qiagen.com

Norway ▪ techservice-nordic@qiagen.com

Singapore ▪ techservice-sg@qiagen.com

Sweden ▪ techservice-nordic@qiagen.com

Switzerland ▪ techservice-ch@qiagen.com

UK ▪ techservice-uk@qiagen.com

USA ▪ techservice-us@qiagen.com

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