October 2010

HotStar HiFidelity PCR Handbook

For sensitive and reliable high-fidelity hot-start PCR

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Contents

Kit Contents

HotStar HiFidelity Polymerase Kit	(100)	(1000)
Catalog no.	**202602**	**202605**
Amount of units	**100**	**1000**
Number of 50 µl PCR reactions	**40**	**400**
HotStar HiFidelity DNA Polymerase	40 µl	2 x 200 µl
HotStar HiFidelity PCR Buffer, 5x, (contains dNTPs)*	400 µl	3 x 1.4 ml
$MgSO_4$, 25 mM	1.2 ml	2 x 1.2 ml
Q-Solution, 5x	400 µl	5 x 2 ml
RNase-Free Water	1.9 ml	8 x 1.9 ml
Handbook	1	1

* Contains 1.5 mM dNTPs, Factor SB, and 7.5 mM $MgSO_4$.

Shipping and Storage

The HotStar HiFidelity Polymerase Kit is shipped on dry ice but retains full activity at room temperature (15–25°C) for 2 weeks.

The HotStar HiFidelity Polymerase Kit, including buffers and reagents, should be stored immediately upon receipt at –20°C in a constant-temperature freezer. When stored under these conditions and handled correctly, the product can be kept at least until the expiration date (see the inside of the kit lid) without showing any reduction in performance.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Product Use Limitations

HotStar HiFidelity DNA Polymerase is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN® products. If you have any questions or experience any difficulties regarding HotStar HiFidelity Polymerase Kit, or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support center at www.qiagen.com/goto/TechSupportCenter or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS 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

Product Specifications

HotStar HiFidelity DNA Polymerase:

HotStar HiFidelity Polymerase is a uniquely modified form of a recombinant 90 kDa DNA polymerase, originally isolated from a novel *Pyrococcus* strain, cloned in *E. coli* (Deoxynucleoside-triphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7). The enzyme possesses a 3' → 5' exonuclease activity, generally referred to as proofreading activity.

One unit of HotStar HiFidelity DNA Polymerase is defined as the amount of enzyme that will incorporate 10 nmol of dNTPs into acid-insoluble material within 30 minutes at 72°C, under the assay conditions described in Quality Control on the following page.

Concentration:	2.5 units/µl
5' → 3' exonuclease activity:	No
3' → 5' exonuclease activity:	Yes
Extra A addition (terminal transferase activity):	Yes
Half-life:	>4 hours at 95°C
Nuclease contamination:	No
Protease contamination:	No
RNase contamination:	No
Storage and dilution buffer:	20 mM Tris·Cl, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.5% (v/v) Nonidet® P-40, 0.5% (v/v) Tween® 20, 50% (v/v) glycerol, stabilizer; pH 9.0 (20°C)

Buffers and reagents:

HotStar HiFidelity PCR Buffer:	5x concentrated. Contains Tris·Cl, KCl, $(NH_4)_2$ SO_4, 7.5 mM $MgSO_4$, bovine serum albumin, Triton® X-100, Factor SB; pH 8.7 (20°C), 1.5 mM dNTPs (dATP, dCTP, dGTP, and dTTP ultrapure quality).
Q-Solution:	5x concentrated
$MgSO_4$ solution:	25 mM
RNase free water:	Ultrapure quality, PCR-grade

Quality Control

Enzyme:	(See quality-control label inside kit lid for lot-specific values.)
Unit assay:	Sonicated herring-sperm DNA (12.5 µg) is incubated with 0.01–0.1 units of HotStar HiFidelity DNA Polymerase in assay buffer (25 mM TAPS [tris-(hydroxymethyl)-methyl-aminopropane-sulfonic acid, sodium salt], pH 9.3 at 20°C; 50 mM KCl; 1 mM DTT; 200 µM of each dNTP; 100 µCi [α-^{32}P] dCTP) at 72°C for 30 minutes. The amount of incorporated dNTPs is determined by precipitation with trichloroacetic acid. HotStar HiFidelity DNA Polymerase is activated by heating for 3 hours at 80°C prior to activity measurement.
Amplification assay:	The amplification efficiency is tested in parallel amplification reactions and is indicated under "Amp".
PCR reproducibility assay:	PCR reproducibility and specificity is tested in parallel amplification reactions. The reactions must yield a single specific product.
Endonuclease activity assay:	Plasmid DNA is incubated with HotStar HiFidelity DNA Polymerase in PCR buffer. Endonuclease activity per unit of enzyme is indicated under "Endo".
RNase activity assay:	RNA is incubated with HotStar HiFidelity DNA Polymerase in PCR buffer. RNase activity per unit of enzyme is indicated under "RNase".
Buffers and Reagents:	
HotStar HiFidelity PCR Buffer, 5x:	Performance in PCR is tested.
Q-Solution, 5x:	Conductivity, pH, sterility, and performance in PCR are tested.
$MgSO_4$, 25 mM:	Conductivity, pH, sterility, and performance in PCR are tested.
RNase-free water:	Conductivity, pH, and RNase activities are tested.

Introduction

HotStar HiFidelity DNA Polymerase is a new hot-start proofreading enzyme uniquely modified to prevent degradation of primers and template during PCR setup, providing highly sensitive and reliable high-fidelity PCR. The kit comes complete with enzyme, buffers, and dNTPs and is ready to use with minimal optimization required. The 5x HotStar HiFidelity PCR Buffer contains a unique additive, Factor SB (patent pending), as well as optimitized dNTPs and $MgSO_4$ concentrations. Factor SB strongly improves sensitivity and reliability of the high-fidelity PCR. The kit also contains Q-Solution, a unique additive enabling efficient amplification of "difficult" (e.g., GC-rich) templates.

Owing to its high proofreading activity, this hot-start enzyme generates PCR products with more than 10 times the accuracy of *Taq* DNA polymerase, providing the ideal tool for all PCR applications that require a low error rate, such as cloning and site-directed mutagenesis.

HotStar HiFidelity DNA Polymerase

HotStar HiFidelity DNA Polymerase was isolated from a novel hyperthermophilic *Pyrococcus* species and cloned into *E. coli*. This recombinant 90 kDa DNA polymerase contains a 3' → 5' exonuclease activity, commonly referred to as "proofreading" activity. This exonuclease activity functions by recognizing and removing incorrectly incorporated deoxynucleotides.

Proofreading activities, however, can also hydrolyze primer and template molecules. Thus, common proofreading polymerases degrade primers during PCR setup causing nonspecific PCR products, smearing, or failure of PCR, especially when only low amounts of starting template are used. HotStar HiFidelity DNA Polymerase has been chemically modified to temporarily inactivate not only the polymerase activity but also the 3' → 5' exonuclease activity of the enzyme. This prevents excessive degradation of primers and template during PCR setup and the initial PCR cycles. Both the polymerase and proofreading activities are easily restored by a 5-minute, 95°C incubation step, which can be readily incorporated into the PCR cycling program. This modification allows convenient room temperature reaction setup without compromising PCR specificity or product yield.

HotStar HiFidelity PCR Buffer

The novel HotStar HiFidelity PCR Buffer contains the new PCR additive Factor SB (patent pending) as well as optimized concentrations of dNTPs and $MgSO_4$. Factor SB is a unique PCR additive, which improves the sensitivity and reliability of the PCR reactions, especially when low amounts of starting template are used.

The preoptimzed formulation provides reliable amplification of specific PCR products with a very low error rate. The buffer accomplishes this by promoting a high ratio of specific-to-nonspecific primer binding during the annealing step in each PCR cycle. Owing to a uniquely balanced combination of KCl and $(NH_4)_2SO_4$, the HotStar HiFidelity 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 therefore often minimal or not required.

The HotStar HiFidelity PCR Buffer contains specifically optimized concentration of dNTPs to provide convenient setup and reliable PCR results. The buffer also maximizes the fidelity of the HotStar HiFidelity DNA Polymerase, which ensures a very low error rate. The increased specificity and minimal optimization makes HotStar HiFidelity PCR buffer ideal for use in any high-fidelity PCR application.

Q-Solution

The HotStar HiFidelity Polymerase Kit is provided 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. Adding Q-Solution to the PCR does not compromise PCR fidelity. For further information, please read the PCR protocol using HotStar HiFidelity DNA Polymerase and Q-Solution, page 16.

Complete kit format

The HotStar HiFidelity Polymerase Kit comes complete and ready to go. No additional reagents or buffers are required.

Cloning PCR products generated using HotStar HiFidelity DNA Polymerase

In contrast to other proofreading DNA polymerases, PCR products generated using HotStar HiFidelity DNA Polymerase can be used directly in TA- or UA-cloning procedures. Cloning efficiency, however, may be slightly lower compared with PCR products generated using *Taq* DNA polymerase (see Figure 1)*.

For efficient cloning we recommend QIAGEN PCR Cloning Kits. Ligation of the PCR products, transformation, and plating of QIAGEN EZ Competent Cells takes place in just 40 minutes.

Read length and sensitivity

Due to the high sensitivity of the HotStar HiFidelity Polymerase Kit, it is possible to amplify fragments with very low amounts of starting material (e.g., as little as 1 ng human genomic DNA). For more details, see Table 18 on page 35). Read lengths of up to 5 kb and 10 kb have been achieved using genomic DNA and lambda DNA respectively, thus indicating the suitability of the HotStar HiFidelity Polymerase kit for any application including the amplification of with longer DNA fragments for cloning.

For very long PCR products of up to 40 kb, we recommend the QIAGEN Long Range PCR Kit (see ordering information).

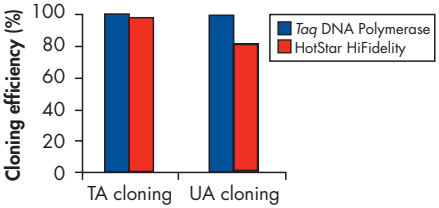
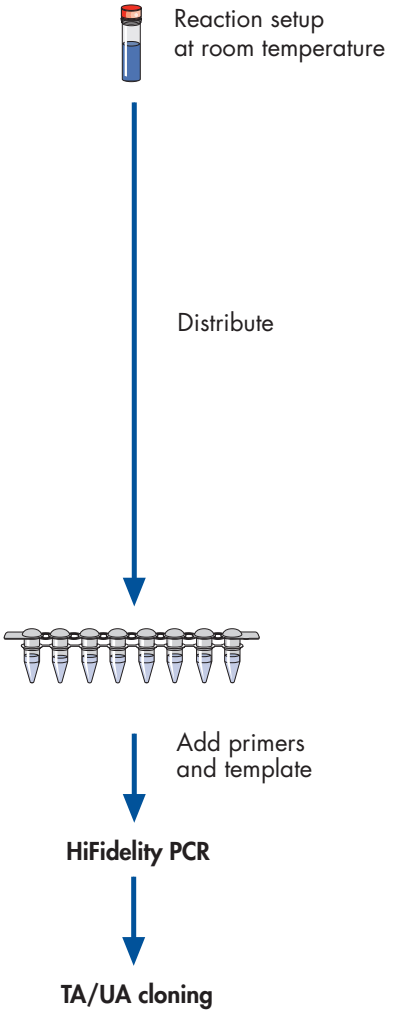


Figure 1. A 955 bp PCR product was amplified from 100 ng of human genomic DNA using the HotStar HiFidelity Polymerase Kit and standard *Taq* DNA Polymerase. 4 µl of each PCR product was cloned using either a commercially available TA or UA cloning kit. Blue/white screening was used to determine the cloning efficiency.

* No addition or blunt end ligation steps need to be performed.

HotStar HiFidelity Procedure



Protocol: Amplification Using HotStar HiFidelity DNA Polymerase

Important points before starting

- HotStar HiFidelity DNA Polymerase requires an **activation step of 5 min at 95°C (see step 6 of this protocol)**.
- The 5x PCR buffer already contains optimized concentrations of dNTPs and $MgSO_4$.
- Use a final primer concentration of 1 µM for each primer.
- Very low amounts of starting template may require lower concentrations of HotStar HiFidelity DNA Polymerase. See Table 19 in Appendix C, page 35.
- To amplify PCR products ≤2kb, use the values marked with a ●; to amplify PCR products 2–5 kb, use the values marked with a ▲. (**Note**: For PCR products >5 kb, see the protocol for amplification of long PCR products on page 21.)
- Follow the optimized protocols. High-Fidelity DNA Polymerases require different cycling conditions to *Taq* DNA Polymerases.

Procedure

1. **Thaw 5x HotStar HiFidelity PCR Buffer, primer solutions, and 25 mM $MgSO_4$ (if required). Mix the solutions completely before use.**
2. **Prepare a reaction mix according to Table 1.**

   It is not necessary to keep reaction vessels on ice since HotStar HiFidelity DNA Polymerase is inactive at room temperature.

   Note: The Mg^{2+} concentration provided by the supplied PCR buffer will produce satisfactory results in most cases. However, in some cases, reactions may be improved by increasing the final Mg^{2+} concentration by 0.5–1.0 mM (see Table 3, page 14).

Table 1. PCR Components (Reaction Mix and Template DNA)

Component	Volume/reaction	Final concentration
Reaction mix		
5x HotStar HiFidelity PCR Buffer (contains dNTPs)*	10 µl	1x
Primer A	Variable	1 µM‡
Primer B	Variable	1 µM‡
HotStar HiFidelity DNA Polymerase (2.5 units/µl)	● 1 µl,† ▲ 2 µl†	● 2.5 units, ▲ 5 units
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	See Table 2
Total volume	**50 µl**	**–**

* Contains optimized concentration of dNTPs and 7.5 mM $MgSO_4$. The Mg^{2+} concentration provided gives successful PCR results in most cases. For potential optimization of Mg^{2+} concentration, please refer to Table 3 on page 14.

† Dependent on expected PCR product length. In general, use ● 1 µl enzyme when amplifying PCR products ● ≤2kb and ▲ 2 µl enzyme when amplifying PCR products ▲ 2–5 kb.

‡ See Table 15 (page 33) for molar conversion ratios.

Table 2. Optimal Amounts of Starting Template from Different Origins

Starting template‡	Optimal range
Human genomic DNA	1 ng – 200 ng
cDNA§	10 ng – 100 ng
Bacterial DNA	10 pg – 10 ng
PCR fragment (1 kb DNA)	10 fg – 1 ng
Plasmid DNA	0.1 ng – 50 ng

‡ If template amounts strongly differ from the given value, refer to Appendix C, Table 19, page 35.

§ Optimal starting template amount depends on the abundance of the respective molecule in your sample. The lower value refers to highly abundant transcripts and the upper values to low abundant transcripts. To amplify long cDNA species, it is strongly recommended to use cDNA generated using oligo-dT primers only.

Table 3. Final Mg^{2+} Concentrations

Final Mg^{2+} concentration in reaction (mM):	1.5	2.0	2.5
Required volume of 25 mM $MgSO_4$ per reaction (µl):	0	1	2

3. **Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes.**

   It is not necessary to keep PCR tubes on ice since HotStar HiFidelity DNA Polymerase is inactive at room temperature.

4. **Add template DNA (see Table 2, page 13) to the individual tubes containing the reaction mix.**

   For two-step RT-PCR, the volume of cDNA added (from the RT reaction) as template should not exceed 10% of the final PCR volume. See Appendix E, page 36, for more information about RT-PCR.

5. **When using a thermal cycler with a heated lid, do not use mineral oil. Proceed directly to step 6. Otherwise, overlay with approximately 50 µl mineral oil.**

6. **Program the thermal cycler according to the manufacturer's instructions.**

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

Table 4. Optimized PCR Cycling Protocol

	Time	Optimized temperature	Comments
Initial activation step:	**5 min**	**95°C**	HotStar HiFidelity DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation:	15 sec	94°C	
Annealing:	1 min	50–68°C	Approximately 5°C below T_m of primers (see Table 15, Appendix B, page 32).
Extension:	● 1 min/kb ▲ 2 min/kb	● 72°C ▲ 68°C	For PCR products of ≤2 kb, use an extension time of ● 1 min per kb DNA. For PCR products 2–5 kb, use ▲ 2 min per kb DNA.*
Number of cycles:	30–45		The cycle number is dependent on the amount of template DNA, see Appendix C, page 34.
Final extension step	10 min	72°C	
End of PCR cycling:	Indefinite	4°C	It is recommended to program a 4°C hold step at the end of the PCR program

* It may be possible to reduce extension time to 1 min per kb.

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

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

Note: For direct cloning of amplified fragments, a TA/UA cloning system can be used. See Appendix J, page 40.

Protocol: Amplification Using HotStar HiFidelity DNA Polymerase 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.

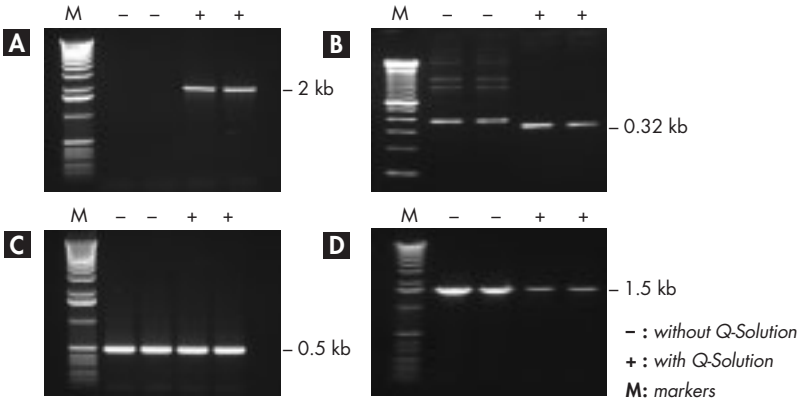
When using Q-Solution, the following effects may be observed depending on the individual PCR assay:

Case A: Q-Solution enables amplification of a reaction which 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

- HotStar HiFidelity DNA Polymerase requires an **activation step of 5 min at 95°C (see step 6 of this protocol)**.
- The 5x PCR buffer already contains optimized concentrations of dNTPs and $MgSO_4$.
- Use a final primer concentration of 1 µM for each primer.
- Very low amounts of starting template may require lower concentrations of HotStar HiFidelity DNA Polymerase. See Table 19 in Appendix C, page 35.
- When using Q-Solution for the first time in a particular primer–template system, it is important to perform parallel amplification reactions with and without Q-Solution.
- To amplify PCR products ≤2 kb, use the values in red (marked with a ●); to amplify PCR products 2–5 kb, use the values in blue (marked with a ▲). (**Note**: For PCR products >5 kb, see the protocol for amplification of long PCR products on page 21.)
- Follow the optimized protocols. High-Fidelity DNA Polymerases require different cycling conditions to *Taq* DNA Polymerases.

Procedure

1. **Thaw 5x HotStar HiFidelity PCR Buffer, primer solutions, Q-Solution, and 25 mM $MgSO_4$ (if required). Mix the solutions completely before use.**
2. **Prepare a reaction mix according to Table 5.**

   It is not necessary to keep PCR tubes on ice since HotStar HiFidelity DNA Polymerase is inactive at room temperature.

   Note: The Mg^{2+} concentration provided by the supplied PCR buffer will produce satisfactory results in most cases. However, in some cases, reactions may be improved by increasing the final Mg^{2+} concentration by 0.5–1.0 mM (see Table 7).

Table 5. PCR Components (Reaction Mix and Template DNA)

Component	Volume/reaction	Final concentration
Reaction mix		
5x HotStar HiFidelity PCR Buffer (contains dNTPs)*	10 µl	1x
5x Q-Solution	10 µl	1x
Primer A	Variable	1 µM‡
Primer B	Variable	1 µM‡
HotStar HiFidelity DNA Polymerase (2.5 units/µl)	● 1 µl,† ▲ 2 µl†	● 2.5 units, ▲ 5 units
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	See Table 6
Total volume	**50 µl**	**–**

* Contains optimized concentration of dNTPs and 7.5 mM $MgSO_4$. The Mg^{2+} concentration provided gives successful PCR results in most cases. For potential optimization of Mg^{2+} concentration, please refer to Table 7 on page 19.

† Dependent on expected PCR product length. In general, use ● 1 µl enzyme when amplifying PCR products ● ≤2kb and ▲ 2 µl enzyme when amplifying PCR products ▲ 2–5 kb.

‡ See Table 15 (page 33) for molar conversion ratios.

Table 6. Optimal Amounts of Starting Template from Different Origins

Starting template‡	Optimal range
Human genomic DNA	1 ng – 200 ng
cDNA§	10 ng – 100 ng
Bacterial DNA	10 pg – 10 ng
PCR fragment (1 kb DNA)	10 fg – 1 ng
Plasmid DNA	0.1 ng – 50 ng

‡ If template amounts strongly differ from the given value, refer to Appendix C, Table 19, page 35.

§ Optimal starting template amount depends on the abundance of the respective molecule in your sample. The lower value refers to highly abundant transcripts and the upper values to low abundant transcripts. To amplify long cDNA species, it is strongly recommended to use cDNA generated using oligo-dT primers only.

Table 7. Final Mg^{2+} Concentrations

Final Mg^{2+} concentration in reaction (mM):	1.5	2.0	2.5
Required volume of 25 mM $MgSO_4$ per reaction (µl):	0	1	2

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes.**

   It is not necessary to keep PCR tubes on ice since HotStar HiFidelity DNA Polymerase is inactive at room temperature.

4. **Add template DNA (Table 6) to the individual tubes containing the reaction mix.**

   For two-step RT-PCR, the volume of cDNA added (from the RT reaction) as template should not exceed 10% of the final PCR volume. See Appendix E, page 36, for more information about RT-PCR.

5. **When using a thermal cycler with a heated lid, do not use mineral oil. Proceed directly to step 6. Otherwise, overlay with approximately 50 µl mineral oil.**

6. **Program the thermal cycler according to the manufacturer's instructions.**

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

Table 8. Optimized PCR Cycling Protocol

	Time	Optimized temperature	Comments
Initial activation step:	**5 min**	**95°C**	HotStar HiFidelity DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation:	15 sec	94°C	
Annealing:	1 min	50–68°C	Approximately 5°C below T_m of primers (see Table 15, Appendix B, page 32).
Extension:	● 1 min/kb ▲ 2 min/kb	● 72°C ▲ 68°C	For PCR products of ≤2 kb, use an extension time of ● 1 min per kb DNA. For PCR products 2–5 kb, use ▲ 2 min per kb DNA.*
Number of cycles:	30–45		The cycle number is dependent on the amount of template DNA, see Appendix C, page 34.
Final extension step	10 min	72°C	
End of PCR cycling:	Indefinite	4°C	It is recommended to program a 4°C hold step at the end of the PCR program

* It may be possible to reduce extension time to 1 min per kb.

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

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

Note: For direct cloning of amplified fragments, a TA/UA cloning system can be used. See Appendix J, page 40.

Protocol: Amplification of Long PCR Products

This protocol has been optimized for amplifying long PCR products (>5 kb)* using a combination of HotStar HiFidelity DNA Polymerase and QIAGEN *Taq* DNA Polymerase or HotStarTaq *Plus* DNA Polymerase (see page 41 for ordering information).

Note: Amplification of long PCR products requires comparably long DNA fragments as template. Amplification of long PCR products is not possible with degraded or highly fragmented DNA.

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, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- QIAGEN *Taq* DNA Polymerase (cat. no. 201203) or HotStarTaq *Plus* DNA Polymerase Kit (cat. no. 203603)
- 10x QIAGEN PCR Buffer (supplied with QIAGEN *Taq* DNA Polymerase or HotStarTaq Plus DNA Polymerase Kit)
- dNTPs, 10 mM each (e.g., dNTP Mix, PCR Grade, cat. no. 201900)

Important points before starting

- Use the 10x PCR Buffer supplied with QIAGEN *Taq* DNA Polymerase or HotStarTaq *Plus* DNA Polymerase in combination with Q-Solution.
- Use a final primer concentration of 0.5 µM for each primer.
- Use a final concentration of 300 µM of each dNTP.
- An extension temperature of 68°C is strongly recommended.
- The denaturation step should not last longer than 10 s.
- Use 0.2 units HotStar HiFidelity DNA Polymerase per reaction. If necessary, dilute HotStar HiFidelity DNA Polymerase in 1x HotStar HiFidelity PCR Buffer.
- This protocol requires an **activation step of only 2 min at 95°C (see step 6 of this protocol)**. This short activation step differs from the values given in the other protocols in this handbook.
- Follow the optimized protocols. High-Fidelity DNA Polymerases require different cycling conditions to *Taq* DNA Polymerases.

* Refers to complex templates (e.g., genomic DNA).

Procedure

1. **Thaw 10x PCR Buffer, dNTP mix, primer solutions, Q-Solution, and 25 mM $MgCl_2$ (if required). Mix the solutions completely before use.**

   Use the PCR Buffer supplied with QIAGEN *Taq* DNA Polymerase or HotStarTaq *Plus* DNA Polymerase.

2. **Prepare a reaction mix according to Table 9.**

   The optimal Mg^{2+} concentration should be determined empirically, but in most cases a concentration of 1.5 mM, as provided in the 1x PCR Buffer, will produce satisfactory results.

   Note: Reaction mix preparation and reaction setup should be performed on ice. If using HotStarTaq *Plus*, reaction setup can be performed at room temperature.

Table 9. PCR Components (Reaction Mix and Template DNA)

Component	Volume/reaction	Final concentration
PCR Buffer,* 10x (supplied with QIAGEN *Taq* DNA Polymerase or HotStarTaq *Plus* DNA Polymerase)	5 µl	1x
Q-Solution, 5x	10 µl	1x
dNTP mix (10 mM of each)	1.5 µl	300 µM of each dNTP
Primer A	Variable	0.5 µM‡
Primer B	Variable	0.5 µM‡
Taq DNA Polymerase or HotStarTaq *Plus* DNA Polymerase	1 µl	5 units
HotStar HiFidelity DNA Polymerase (diluted)†	1 µl	0.2 units
RNase free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	See Table 10
Total volume	50 µl	

* Contains 15 mM $MgCl_2$. The Mg^{2+} concentration provided gives successful PCR results in most cases. For potential optimization of Mg^{2+} concentration, please refer to Table 11 on page 23.

† Dilute enzyme in 1x HotStar HiFidelity PCR Buffer to a concentration of 0.2 units/µl.

‡ See Table 15 (page 33) for molar conversion ratios.

Table 10. Optimal Amounts of Starting Template from Different Origins

Starting template*	Optimal range
Human genomic DNA	1 ng – 200 ng
cDNA†	10 ng – 100 ng
Bacterial DNA	10 pg – 10 ng
PCR fragment (1 kb DNA)	10 fg – 1 ng
Plasmid DNA	0.1 ng – 50 ng

* If template amounts strongly differ from the given value, refer to Appendix C, Table 19, page 35.

† Optimal starting template amount depends on the abundance of the respective molecule in your sample. The lower value refers to highly abundant transcripts and the upper values to low abundant transcripts. To amplify long cDNA species, it is strongly recommended to use cDNA generated using oligo-dT primers only.

Table 11. Final Mg^{2+} Concentrations

Final Mg^{2+} concentration in reaction (mM):	**1.5**	**2.0**	**2.5**	**3.0**
Required volume of 25 mM $MgCl_2$ per reaction (µl):	**0**	**1**	**2**	**3**

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes.**
   It is recommended to keep the PCR tubes on ice before placing in the thermal cycler.
4. **Add template DNA (see Table 9) to the individual tubes containing the reaction mix.**
   For two-step RT-PCR, the volume of cDNA added (from the RT reaction) as template should not exceed 10% of the final PCR volume. See Appendix E, page 36, for more information about RT-PCR.
5. **When using a thermal cycler with a heated lid, do not use mineral oil. Proceed directly to step 6. Otherwise, overlay with approximately 50 µl mineral oil.**
6. **Program the thermal cycler according to the manufacturer's instructions.**
   Each PCR program must start with an initial heat activation step at 95°C for 2 min.

Table 12. Optimized PCR Cycling Protocol

	Time	Optimized temperature	Comments
Initial activation step:	2 min	95°C	HotStar HiFidelity DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation:	10 s	94°C	
Annealing:	1 min	50–68°C	Approximately 5°C below T_m of primers (see Table 16, Appendix B, page 32).
Extension:	1 min/kb	68°C	Use an extension time of approximately 1 min per kb DNA
Number of cycles:	25–45		The cycle number is dependent on the amount of template DNA, (see Appendix C, page 34).
End of PCR cycling:	Indefinite	4°C	

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

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

Note: For direct cloning of amplified fragments, a TA/UA cloning system can be used. See Appendix J, page 39.

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)	HotStar HiFidelity DNA Polymerase not activated	Check if PCR was started with an initial incubation step at 95°C for 5 min. For amplification of long PCR products, use a 2 min activation step as recommended in the protocol on page 21.
b)	Pipetting error or missing reagent	Repeat the PCR. Check the concentrations and storage conditions of reagents, including primers and template.
c)	Insufficient starting template	Increase amount of starting template used in PCR. Use the values given in Tables 2, 6, or 10 in the relevant protocol as a starting point. Also, refer to the information in Appendix A, page 30, concerning starting template amount and corresponding cycle numbers and enzyme concentration. If necessary, perform a second round of PCR using a nested-primer approach (see Appendix D, page 36).
d)	PCR conditions not optimal	Using the same cycling conditions, repeat the PCR using Q-Solution. Follow the protocol on page 16.
f)	Primer concentration not optimal	Use a concentration of 1 µM of each primer. It is not recommended to use primer concentrations lower than 0.5 µM. For calculation of primer concentration, refer to Appendix B, pages 31–34.
g)	Extension step not optimal	Increase extension time in increments of 1 min. Ensure that an extension time of ▲ 2 min per kb at 68°C was used for PCR products of >2 kb.
h)	Enzyme concentration too low	For PCR fragments ● ≤2 kb, use ● 1 µl Hot Start HiFidelity DNA Polymerase per 50 µl reaction. For PCR fragments of ▲ 2–5 kb, use ▲ 2 µl of enzyme per 50 µl reaction. If necessary, increase amount of HotStar HiFidelity DNA Polymerase (in 0.5-unit steps). See also Table 19 in the Appendix C, page 35.

		Comments and suggestions
i)	Insufficient number of cycles	Increase the number of cycles in steps of 5 cycles (see Appendix C, page 34). Refer to the information in the protocols and Appendix A, page 30, concerning starting template amount and corresponding cycle numbers and enzyme concentration.
j)	Incorrect PCR volume	Do not use PCR volumes greater than 50 µl.
k)	Problems with starting template	Check the concentration, storage conditions, and quality of starting template (see Appendix A, pages 30–31). If necessary, make new serial dilutions of template nucleic acid from stock solutions. Repeat the PCR using the new dilutions.
m)	Incorrect annealing temperature or time	Use the optimised annealing time of 60 s. Use an annealing temperature 5°C below the T_m of your primers. See Appendix B, page 31, for details on how to determine the annealing temperature of your primers. Difficulties in determining the optimal annealing temperature can often be overcome by performing a gradient PCR (see Appendix F, page 37).
n)	Incorrect denaturation temperature or time	Denaturation should be at 94°C for 15 sec. Ensure that the initial 5-min 95°C incubation step was performed as described in step 6 of the PCR protocols (pages 14, 19, and 23).
l)	Mg^{2+} concentration not optimal	Perform PCR with different final concentrations of Mg^{2+} from 1.5 to 3 mM (in 0.5 mM steps) using the 25 mM $MgSO_4$ solution provided (see Table 3, page 14).
p)	Primer design not optimal	Review primer design (see Appendix B, pages 31–34).
q)	RT reaction error	For RT-PCR, take into consideration the efficiency of the reverse transcriptase reaction, which averages from 10 to 30%. The use of oligo-dT primers in the reverse-transcription reaction is preferred over random primers for amplification of cDNA species of >1kb. (See *Omniscript® Reverse Transcription Handbook* for more details). The added volume of reverse transcriptase reaction should not exceed 10% of the final PCR volume (see Appendix E, page 36).

r)	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. This may decrease the yield of PCR product.
t)	Problems with the thermal cycler	Check the power to the thermal cycler and that the thermal cycler has been correctly programmed.

Product is multi-banded

a	PCR cycling conditions not optimal	Using the same cycling conditions, repeat the PCR using Q-Solution. Follow the protocol on page 16.
b)	Annealing temperature too low	Use an annealing temperature 5°C below the T_m of your primers. See Appendix B, page 31, for details on how to determine the annealing temperature of your primers. Use the optimised annealing time of 60 s. Difficulties in determining the optimal annealing temperature can often be overcome by performing a gradient PCR (see Appendix F, page 38).
c)	Extension time too short	Increase extension time in increments of 1 min. Ensure that an extension time of ▲ 2 min per kb at 68°C was used for PCR products of >2 kb. Include a final extension step of 10 min at 72°C.
d)	Enzyme concentration too low	For PCR fragments ● ≤2 kb, use ● 1 µl Hot Start HiFidelity DNA Polymerase per 50 µl reaction. For PCR fragments of ▲ 2–5 kb, use ▲ 2 µl of enzyme per 50 µl reaction. If necessary, increase amount of Hot Start HiFidelity DNA Polymerase (in 0.5-unit steps). See also Table 19 in the Appendix C, page 35.
g)	Primer concentration not optimal or primers degraded	Use a concentration of 1 µM of each primer. It is not recommended to use primer concentrations lower than 0.5 µM. For calculation of primer concentration, refer to Appendix B, pages 31–34. Particularly when performing highly sensitive PCR, check for possible degradation of the primers on a denaturing polyacrylamide gel.
h)	Primer design not optimal	Review primer design (see Appendix B, pages 31–34).

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

		Comments and suggestions
f)	Mg^{2+} concentration not optimal	Perform PCR with different final concentrations of Mg^{2+} from 1.5 to 3 mM (in 0.5 mM steps) using the 25 mM $MgSO_4$ solution provided (see Table 3, page 14).
Product is smeared		
a)	Insufficient starting template	Increase amount of starting template used in PCR. Use the values given in Tables 2, 6, or 10 in the relevant protocol as a starting point. Also, refer to the information in Appendix A, page 30, concerning starting template amount and corresponding cycle numbers and enzyme concentration.
b)	Extension time too short	Increase extension time in increments of 1 min. Ensure that an extension time of ▲ 2 min per kb at 68°C was used for PCR products of >2 kb.
d)	Incorrect enzyme concentration	Check if the enzyme concentration specified in the respective protocol was used. For PCR fragments ● ≤2 kb, use ● 1 µl Hot Start HiFidelity DNA Polymerase per 50 µl reaction. For PCR fragments of ▲ 2–5 kb, use ▲ 2 µl of enzyme per 50 µl reaction. Refer to the information in Appendices A (page 30) and C (page 34) concerning starting template amount and corresponding cycle numbers and enzyme concentration.
e)	Incorrect PCR volume	Do not use PCR volumes greater 50 µl.
f)	Mg^{2+}concentration not optimal	Perform PCR with different final concentrations of Mg^{2+} from 1.5–3.0 mM (in 0.5 mM steps) using the 25 mM $MgSO_4$ solution provided (see Table 3, page 14).
g)	Primer design not optimal	Review primer design (see Appendix B, page 31).
Poor PCR fidelity		
a)	Insufficient starting template	Increase amount of starting template used in PCR. Use the values given in Tables 2, 6, or 10 in the relevant protocol as a starting point. Also, refer to the information in Appendix A, page 30, concerning starting template amount and corresponding cycle numbers and enzyme concentration.

	Comments and suggestions
b) Mg^{2+} concentration too high	Fidelity of PCR is generally affected by the Mg^{2+} concentration. Optimal PCR fidelity with HotStar HiFidelity DNA Polymerase is achieved with a Mg^{2+} concentration of 1.5 to 2.5 mM. Higher Mg^{2+} concentration will lead to lowered fidelity.
Protocol to amplify long PCR products	
dNTP concentration not optimal	Ensure that a dNTP concentration of 300 µM of each dNTP was used. Increasing the dNTP concentration might lower the PCR fidelity. Ensure that fresh and intact dNTPs of sufficiently high quality are used (e.g., dNTP Mix, PCR Grade, cat. no. 201900).
Detection of PCR products using DNA Lab Chip Kits and the Agilent® Bioanalyzer	
Poor chip performance	Perform a 1:10 dilution of your PCR products in water or 10 mM Tris (pH 8.3–8.7) before loading them on a DNA Lab Chip. This is necessary because of detergents present in the 5x HotStar HiFidelity PCR Buffer*.

* If using the QIAxcel System for detection, dilution of PCR products is not necessary.

Appendix A: Starting Template

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, ensuring the highest-quality templates for PCR, for example 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 QIAprep, QIAamp, DNeasy, and RNeasy products, contact one of our Technical Service Departments (see back cover) or visit www.qiagen.com .

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 13 and 14 respectively. For optimal template amounts to be used with HotStar HiFidelity DNA Polymerase, see Tables 18 and 19, page 35.

Table 13. 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 comprehensive brochure *"Critical success factors and new technologies for PCR and RT-PCR"*. 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 14. Molar Conversions for Nucleic Acid Templates

Nucleic acid	Size	pmol/µg	Molecules/µg
1 kb DNA	1000 bp	1.52	9.1×10^{11}
2 kb DNA	2000 bp	0.76	4.6×10^{11}
pUC19 DNA	2686 bp	0.57	3.4×10^{11}
pBluescript® II DNA	2961 bp	0.52	3.1×10^{11}
pBR322	4361 bp	0.35	2.1×10^{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 15, page 32.

Table 15. General Guidelines for Standard PCR Primers

Length:	20–35 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., OLIGO 6, Rychlik, 1999) or web-based tools such as Primer3, Steve Rosen and Helen Skaletsky, 2000 (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), can be used for primer design.

Table continues on next page.

Table 15 (continued)

Concentration:

- Spectrophotometric conversion for primers: 1 A_{260} unit = 20–30 µg/ml
- Molar conversions:

Primer length	pmol/µg	50 pmol
20mer	152	329 ng
25mer	121	413 ng
30mer	101	495 ng
35mer	92	543 ng

- **For most applications, use 1 µM of each primer (50 pmol of each primer per 50 µl reaction).**

Storage:

Lyophilized primers should be dissolved in a small volume of TE* (10 mM Tris·Cl, 1mM EDTA, pH 8.0) to obtain a 50 or 100 µM stock solution.
Prepare small aliquots of working solutions containing 10 µM (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*; please call one of the QIAGEN Technical Service departments or local distributor for a protocol (see back cover) or visit www.qiagen.com .

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

Degenerate PCR primers

Occasionally, the exact nucleotide sequence of the target-template DNA will not be known, for instance when it has been deduced from an amino acid sequence. To enable such templates to be amplified by PCR, degenerate primers can be used. These are actually mixtures of several primers whose sequences differ at the position that correspond to the uncertainties in the template sequence.

Hot-start PCR using HotStarTaq DNA Polymerase often improves the specificity of PCR amplifications that employ degenerate primers by reducing the formation of non-specific PCR products and primer–dimers. Table 16 gives recommendations for further optimizing PCR using degenerate primers. Table 17 shows the codon redundancy of each amino acid.

Table 16. Guidelines for Design and Use of Degenerate Primers

Sequence:	■ Avoid degeneracy in the 3 nucleotides at the 3' end. ■ If degeneracy cannot be avoided at the 3'-terminal bases, the oligonucleotide supplier can synthesize primers with one phosphorothioate bond between the two 3'-terminal nucleotides. ■ If possible, use Met- or Trp-encoding triplets at the 3' end. ■ To increase primer–template binding efficiency, reduce degeneracy by allowing mismatches between the primer and template, especially towards the 5' end (but not at the 3' end). ■ Try to design primers with less than 4-fold degeneracy at any given position.
Concentration:	■ Begin PCR with a primer concentration of 1 µM. ■ In case of poor PCR efficiency, increase primer concentrations in increments of 0.1 µM until satisfactory results are obtained.

Table 17. Codon Redundancy

Amino acid	Number of codons
Met, Trp	1
Cys, Asp, Glu, Phe, His, Lys, Asn, Gln, Tyr	2
Ile	3
Ala, Gly, Pro, Thr, Val	4
Leu, Arg, Ser	6

Appendix C: Number of PCR Cycles

When using HotStar HiFidelity DNA Polymerase, a typical cycling program consists of 25–50 cycles, depending on the number of copies of the starting template and the enzyme concentration. Table 18 provides general guidelines to determine the number of copies; Table 19 provides general guidelines for choosing the number of cycles and the enzyme concentration, depending on the amount of starting template and the expected length of the PCR product.

Table 18. General Guidelines for Choosing the Number of Copies of Template

Number of copies of starting template*	1 kb DNA	E. coli DNA†	Human genomic DNA†
10–100	0.01–0.11 fg	0.05–0.56 pg	36–360 pg
100–1000	0.11–1.1 fg	0.56–5.56 pg	0.36–3.6 ng
$1 \times 10^3 - 5 \times 10^4$	1.1–55 fg	5.56–278 pg	3.6–179 ng
$5 \times 10^4 - 2 \times 10^5$	55 fg – 0.11 pg	278 pg – 2.8 ng	179 ng – 537 ng‡
$2 \times 10^5 - 2 \times 10^6$	0.11 pg – 1.1 ng	2.8 ng – 28 ng	N. R.

* Refer to Table 14 (page 31) to calculate the number of molecules for template DNA different than the examples given. 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%. Use oligo dT primers for cDNA synthesis for all PCR fragments >1 kb.

† Refers to single-copy genes.

‡ The recommended upper limit for human genomic DNA is approx. 500 ng.

N. R.: Not recommended to use such high amounts of starting template.

Table 19. General Guidelines for Choosing the Number Cycles and Enzyme Concentration for Different Amounts of Starting Template

Number of copies of starting template	Units Hot Start HiFidelity DNA Polymerase* ● Fragments <2kb	▲ Fragments 2–5 kb	Number of cycles
10–100	0.5–1.0	0.5–1.5	50
100–1000	0.5–1.5	1.0–1.5	45–50
$1 \times 10^3 - 1 \times 10^4$	1.5–2.5	1.5–2.5	40–45
$1 \times 10^4 - 5 \times 10^4$	1.5–2.5	1.5–2.5	35–40
$5 \times 10^4 - 2 \times 10^5$	2.5	5.0	35–40
$2 \times 10^5 - 2 \times 10^6$	2.5	5.0	30–35
$2 \times 10^6 - 2 \times 10^7$†	2.5	5.0	25–30

Refer to Table 18 to calculate starting copy number for your template nucleic acid.

Use the higher number of PCR cycles for maximum PCR product yield.

* To amplify PCR products ≤2 kb, use the values in red (marked with a ●). To amplify PCR products 2–5 kb, use the values in blue (marked with a ▲).

† Only recommended for low complexity templates such as plasmid DNA or lambda DNA.

Appendix D: Nested PCR

If PCR sensitivity is too low, a nested PCR method can increase PCR product yield. Nested PCR involves two rounds of amplification reactions. The first-round PCR is performed according to the PCR Protocol using HotStar HiFidelity DNA Polymerase. Subsequently, an aliquot of the first-round PCR product, for example 1 µl of a 1-in-10^3 – 10^4 dilution, is subjected to a second round of PCR. The second-round PCR is performed with two new primers that hybridize to sequences internal to the first-round primer–target sequences. In this way, only specific first-round PCR products (and not nonspecific products) will be amplified in the second round. Alternatively, it is possible to use one internal and one first-round primer in the second PCR; this method is referred to as semi-nested PCR.

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 transcriptase 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 is reverse transcribed into cDNA. To amplify cDNA species >1 kb, it is recommended to use oligo dT primers for cDNA synthesis. Use of random primes may result in relatively short cDNA molecules, which may limit the amplification of fragments >1 kb. 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 10–15% of the total PCR volume. General guidelines as well as guidelines on the amount of cDNA required to amplify high, medium, or low abundant transcripts are presented in Table 20.

For performing cDNA amplification of very long fragments, of up to 12.5 kb, we recommend the QIAGEN Long Range 2 Step RT-PCR Kit (see ordering information on page 40).

Table 20. General Guidelines to Amplify cDNA Molecules in RT-PCR

RNA purification:	■ Intact RNA is a prerequisite to amplify cDNA molecules in RT-PCR. The integrity of the RNA is especially important when you plan to analyze large fragments (e.g., amplification of complete cDNAs for protein expression. QIAGEN offers the RNeasy system for total RNA isolation.
Reverse transcription:	To amplify cDNA species >1 kb it is recommended to use oligo dT primers for reverse transcription. QIAGEN offers 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 oligo dT primer, 12–18mer (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 reaction mix. Typically, 10 to 100 ng cDNA will give satisfactory results. For low abundant transcripts, it is recommended to start with approximately 100 ng cDNA, wherase for for highly abundant transcripts, low amounts (down to 1 ng–100 pg) may be sufficient. ■ Continue with step 5 in the PCR protocols.

* For further information about RNeasy, Long Range PCR and Omniscript products, contact your local QIAGEN Technical Services or distributor (see back cover) or visit www.qiagen.com .

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

Appendix F: Gradient PCR

Many thermal cyclers have a temperature-gradient function. Using this function, it is possible to easily determine optimal annealing temperatures by generating a temperature gradient across the heating block for the annealing step. If your primers conform to the criteria specified in Appendix B on page 31, we recommended using a gradient program that includes a temperature range from 50–70°C. In order to determine optimal annealing conditions, prepare 3 identical reactions and place in the block positions that most closely correspond to the temperatures 1°C, 5°C, and 8°C below the calculated T_m of your primers.

Appendix G: Touchdown PCR

Touchdown PCR uses a cycling program with varying annealing temperatures. It is a useful method to increase the specificity of PCR. The annealing temperature in the initial cycle should be 5–10°C above the T_m of the primers. In subsequent cycles, the annealing temperature is decreased in steps of 1–2°C/cycle until a temperature is reached that is equal to, or 2–5°C below, the T_m of the primers. Touchdown PCR enhances the specificity of the initial primer–template duplex formation and hence the specificity of the final PCR product.

To program your thermal cycler for touchdown PCR, you should refer to the manufacturer's instructions.

Appendix H: 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) or visit www.qiagen.com .

Appendix I: 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 reaction 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 reaction mix. Use of pipet tips with hydrophobic filters is strongly recommended.
- Prepare and freeze small aliquots of primer solutions and dNTP mix. Use of fresh RNase-free water 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 RNase-free 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.

Appendix J: Cloning of PCR Products

In contrast to other proofreading DNA polymerases, PCR products generated using HotStar HiFidelity DNA Polymerase can be used directly in TA- or UA-cloning procedures. Cloning efficiency, however, may be slightly lower compared with PCR products generated using *Taq* DNA polymerase.

For efficient cloning we recommended QIAGEN PCR Cloning Kits. Ligation of the PCR product, transformation, and plating of QIAGEN EZ Competent Cells takes place in just 40 minutes.

* 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 material safety data sheets (MSDSs), available from the product supplier.

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
HotStar HiFidelity Polymerase Kit (1000 U)	1000 units HotStar HiFidelity DNA Polymerase, 5x HotStar HiFidelity PCR Buffer (inc. dNTPs),* 5x Q-Solution, 25 mM $MgSO_4$, RNase-Free Water	202605
Related products		
HotStarTaq *Plus* DNA Polymerase — for highly specific hot-start PCR without optimization		
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 DNA Polymerase — for robust amplification in all applications		
HotStarTaq DNA Polymerase (250 U)*‡	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer,† 5x Q-Solution, 25 mM $MgCl_2$	203203
***Taq* DNA Polymerase — for standard and specialized PCR applications**		
Taq DNA Polymerase (250 U)‡	250 units *Taq* DNA Polymerase, 10x PCR Buffer,† 5x Q-Solution, 25 mM $MgCl_2$	201203
QIAGEN LongRange PCR Kit — for reliable LongRange PCR		
QIAGEN LongRange PCR Kit (20)‡	For 20 x 50 µl reactions: LongRange PCR Enzyme Mix (40 U), LongRange PCR Buffer, 5x Q-Solution, RNase-Free Water, 10 mM dNTPs	206401
QIAGEN LongRange RT-PCR Kit — for reliable 2Step RT-PCR		
QIAGEN LongRange 2Step RT-PCR Kit (20)‡	For 20 x 50 µl PCRs: Reverse transcription step (10 x 20 µl reactions) - LongRange Reverse Transcription Enzyme, Buffer, dNTPs, Oligo-dT, RNase Inhibitor, RNase-Free Water; PCR step - QIAGEN LongRange PCR Kit (see above)	205920

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

† Contains 15 mM $MgCl_2$.

‡ Larger kit sizes available; see www.qiagen.com .

Ordering Information

Product	Contents	Cat. no.
TopTaq DNA Polymerase — for highly reliable end-point PCR with unrivalled ease-of-use		
TopTaq DNA Polymerase (250)*	250 units TopTaq DNA Polymerase, 10x PCR Buffer†, CoralLoad Concentrate, 5x QSolution, 25 mM $MgCl_2$	200203
QIAGEN PCR Cloning Kits — for direct cloning of PCR products generated by Taq and other non-proofreading DNA polymerases		
QIAGEN PCR Cloning Kit(10)*	For 10 reactions: 2x Ligation Master Mix (50 µl), pDrive Cloning Vector (0.5 µg), distilled water (1.7 ml)	231122
QIAGEN PCR Cloning*plus* Kit (10)*	For 10 reactions: 2x Ligation Master Mix (50 µl), pDrive Cloning Vector (0.5 µg), distilled water (1.7 ml), QIAGEN EZ Competent Cells (10 tubes, 50 µl each), SOC medium (2 x 1.9 ml)	231222
RNeasy Mini Kit — for purification of up to 100 µg total RNA from animal cells or tissues, yeast, or bacteria		
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
Omniscript RT Kit — for standard reverse transcription with any amount of RNA from 50 ng to 2 µg per reaction		
Omniscript RT Kit (10)*	For 10 reverse-transcription reactions: 40 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix (contains 5 mM each dNTP), RNase-free water	205110
MinElute PCR Purification Kit — for purification of PCR products (70 bp to 4 kb) in low elution volumes		
MinElute PCR Purification Kit (50)*	50 MinElute Spin Columns, Buffers, Collection Tubes (2 ml)	28004

* Larger kit sizes available; see www.qiagen.com .

† Contains 15 mM $MgCl_2$.

Ordering Information

Product	Contents	Cat. no.
QIAquick PCR Purification Kit — for purification of PCR products (100 bp to 10 kb)		
QIAquick PCR Purification Kit (50) *	For purification of 50 PCR reactions: 50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28104
QIAxcel System — for effortless automated DNA fragment and RNA analysis		
QIAxcel System	Capillary electrophoresis device, including computer, and BioCalculator Analysis software; 1-year warranty on parts and labor	9001421
QIAxcel Kits — for fast high-resolution capillary electrophoresis		
QIAxcel DNA High Resolution Kit (1200)	QIAxcel DNA High Resolution Gel Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips	929002
QIAxcel DNA Screening Kit (2400)	QIAxcel DNA Screening Gel Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips	929004
QIAxcel DNA Large Fragment Kit (600)	QIAxcel DNA Large Fragment Gel Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips	929006

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