

HotStarTaq[®] *Plus* PCR Handbook

HotStarTaq *Plus* DNA Polymerase

HotStarTaq *Plus* Master Mix Kit

For highly specific hot-start PCR
without optimization



QIAGEN Sample and Assay Technologies

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- microRNA research and RNAi
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Kit Contents

HotStarTaq <i>Plus</i> DNA Polymerase	(250 units)	(1000 units)	(5000 units)	(25000 units)
Catalog no.	203603	203605	203607	203609
HotStarTaq <i>Plus</i> DNA Polymerase	250 units	4 x 250 units	1 x 5000 units	100 x 250 units
PCR Buffer, 10x*	1.2 ml	4 x 1.2 ml	1 x 22 ml	100 x 1.2 ml
CoralLoad PCR Buffer, 10x*	1.2 ml	4 x 1.2 ml	1 x 22 ml	100 x 1.2 ml
Q-Solution, 5x	2 ml	4 x 2 ml	1 x 40 ml	100 x 2 ml
MgCl ₂ , 25 mM	1.2 ml	4 x 1.2 ml	1 x 22 ml	100 x 1.2 ml
Handbook	1	1	1	1

* Contains 15 mM MgCl₂.

HotStarTaq <i>Plus</i> Master Mix Kit	(250 units)	(1000 units)	(2500 units)
Catalog no.	203643	203645	203646
Reaction volume (1 unit/reaction)	20 µl	20 µl	20 µl
HotStarTaq <i>Plus</i> Master mix [†]	3 x 0.85 ml	12 x 0.85 ml	1 x 25 ml
CoralLoad Concentrate, 10x	1 x 0.55 ml	4 x 0.55 ml	1 x 5.5 ml
RNase-Free Water	2 x 1.9 ml	8 x 1.9 ml	2 x 20 ml
Handbook	1	1	1

[†] Contains HotStarTaq *Plus* DNA Polymerase, PCR Buffer with 3 mM MgCl₂, and 400 µM each dNTP.

Shipping and Storage

HotStarTaq *Plus* DNA Polymerase is shipped on dry ice but retains full activity at room temperature (15–25°C) for 1 week.

HotStarTaq *Plus* Master Mix Kit is shipped on dry ice but retains full activity at room temperature (15–25°C) for 3 days.

HotStarTaq *Plus* DNA Polymerase and HotStarTaq *Plus* PCR Master Mix, 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, this product can be kept at least until the expiration date (see the inside of the kit lid) without showing any reduction in performance.

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 HotStarTaq *Plus* DNA Polymerase, HotStarTaq *Plus* Master Mix 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).

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

HotStarTaq *Plus* DNA Polymerase and HotStarTaq *Plus* Master Mix Kit are intended for molecular biology applications. These products are 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 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

Enzyme:

HotStarTaq *Plus* DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*, cloned in *E. coli*. (Deoxynucleoside-triphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7).

Concentration:	5 units/ μ l
Substrate analogs:	dNTP, ddNTP, fluorescent dNTP/ddNTP
Extension rate:	2–4 kb/min at 72°C
Half-life:	10 min at 97°C; 60 min at 94°C
5'–3' exonuclease activity:	Yes
Extra A addition:	Yes
3'–5' exonuclease activity:	No
Nuclease contamination:	No
RNase contamination:	No
Self-priming activity:	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% glycerol (v/v), stabilizer; pH 9.0 (20°C).

Buffers and reagents:

PCR Buffer:	10x concentrated. Contains Tris-Cl, KCl, $(\text{NH}_4)_2\text{SO}_4$, 15 mM MgCl_2 ; pH 8.7 (20°C).
CoralLoad PCR Buffer:	10x concentrated. Contains Tris-Cl, KCl, $(\text{NH}_4)_2\text{SO}_4$, 15 mM MgCl_2 , gel loading reagent, orange dye, red dye; pH 8.7 (20°C).
Q-Solution:	5x concentrated
MgCl_2 solution:	25 mM
HotStarTaq <i>Plus</i> Master Mix:	2x concentrated. Contains HotStarTaq <i>Plus</i> DNA Polymerase, PCR Buffer (with 3 mM MgCl_2), and 400 μ M each dNTP.
CoralLoad Concentrate:	10x concentrated. Contains gel loading reagent, orange dye, red dye.

Quality Control

Enzyme:	(See quality-control label inside kit lid for lot-specific values.)
Amplification efficiency assay:	The amplification efficiency is tested in parallel amplification reactions and is indicated under "Amp".
PCR reproducibility assay:	PCR reproducibility and specificity are tested in parallel amplification reactions. The reactions must yield a single specific product.
Exonuclease activity assay:	Linearized plasmid DNA is incubated with HotStarTaq <i>Plus</i> DNA Polymerase in PCR Buffer. Exonuclease activity per unit of enzyme is indicated under "Exo".
Endonuclease activity assay:	Plasmid DNA is incubated with HotStarTaq <i>Plus</i> DNA Polymerase in PCR Buffer. Endonuclease activity per unit of enzyme is indicated under "Endo".
RNase activity assay:	RNA is incubated with HotStarTaq <i>Plus</i> DNA Polymerase in PCR Buffer. RNase activity per unit of enzyme is indicated under "RNase".
Self-priming activity assay:	Assays are performed under standard PCR conditions, without primers, using HotStarTaq <i>Plus</i> DNA Polymerase and human genomic DNA (purified with the QIAamp® DNA Blood Mini Kit). The absence of PCR product is indicated by "No" under "Self-priming".

Buffers and Reagents:

PCR Buffer, 10x:	Conductivity, pH, total aerobic microbial count, and performance in PCR are tested.
CoralLoad PCR Buffer, 10x:	Conductivity, pH, dye concentrations, and performance in PCR are tested.
CoralLoad Concentrate, 10x:	Conductivity and dye concentrations are tested.
Q-Solution, 5x:	Conductivity, pH, and performance in PCR are tested.
MgCl ₂ , 25 mM:	Conductivity and total aerobic microbial count are tested.

HotStarTaq *Plus* Master Mix Kit:

PCR reproducibility assay:	The PCR reproducibility assay described above is performed in parallel using HotStarTaq <i>Plus</i> Master Mix and using the separate reagents with the same lot numbers.
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Introduction

HotStarTaq *Plus* DNA Polymerase has been developed by QIAGEN to provide the unrivaled performance of HotStarTaq DNA Polymerase with a shortened activation time of just 5 minutes. The combination of HotStarTaq *Plus* DNA Polymerase and the unique QIAGEN PCR Buffer or the new CoralLoad PCR Buffer minimizes nonspecific amplification products, primer–dimers, and background. It is ideal for amplification reactions involving complex genomic or cDNA templates, very low-copy targets, or multiple primer pairs. HotStarTaq *Plus* DNA Polymerase makes highly specific hot-start PCR simple and easy, with minimal optimization required. The HotStarTaq Master Mix Kit provides all of the benefits of HotStarTaq *Plus* DNA Polymerase and more, including dNTPs and reduced number of pipetting steps — reducing the risk of contamination.

HotStarTaq *Plus* DNA Polymerase

HotStarTaq *Plus* DNA Polymerase 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. In contrast to the 15-minute, 95°C incubation step required by HotStarTaq DNA Polymerase, 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 high PCR specificity 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.

CoralLoad PCR Buffer and CoralLoad Concentrate

HotStarTaq *Plus* DNA Polymerase is supplied with new CoralLoad PCR Buffer, which has all of the advantages of QIAGEN PCR Buffer (also supplied) but can also be used to directly load the PCR products onto an agarose gel without the need to add a gel loading buffer. CoralLoad PCR Buffer provides the same high PCR specificity and minimal reaction optimization as conventional QIAGEN PCR Buffer. Additionally, it contains 2 marker dyes — an orange dye and a red dye — that facilitate estimation of DNA migration distance and optimization of agarose gel run time. HotStarTaq *Plus* Master Mix contains CoralLoad Concentrate, which contains a gel loading reagent and 2 gel tracking dyes. When using CoralLoad Concentrate, the PCR products can be directly loaded onto an agarose gel without prior addition of loading buffer. CoralLoad Concentrate can be added to the PCR without affecting amplification sensitivity or specificity. PCR fragments amplified in the presence of CoralLoad PCR Buffer or Concentrate have been successfully tested for cloning and restriction digestion without prior purification.

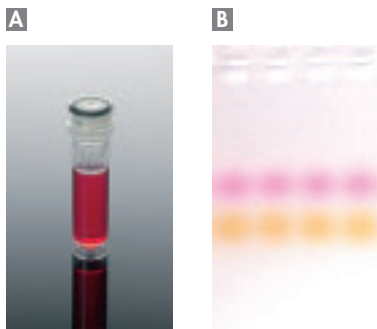


Figure 1. CoralLoad PCR Buffer and Concentrate. CoralLoad PCR Buffer and Concentrate **A** contains gel-tracking dyes **B** enabling immediate loading of PCR products and easy visualization of DNA migration.

QIAGEN PCR Buffer

The innovative QIAGEN PCR Buffer facilitates the amplification of specific PCR products. During the annealing step of every PCR cycle, the buffer allows a high ratio of specific-to-nonspecific primer binding. Owing to a uniquely balanced combination of KCl and $(\text{NH}_4)_2\text{SO}_4$, 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.

Q-Solution

HotStarTaq *Plus* DNA Polymerase 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. For further information, please read the Protocol "PCR Using HotStarTaq *Plus* DNA Polymerase and Q-Solution", page 19.

* 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).

Specificity and sensitivity

HotStarTaq *Plus* DNA Polymerase in combination with original QIAGEN PCR Buffer or new CoralLoad PCR Buffer with its balanced potassium and sodium salts promotes specific primer–template annealing and simultaneously reduces non-specific annealing. Maximum yields of specific products are obtained even when using extremely low template amounts. See single-cell PCR protocol, page 24.

Downstream applications

HotStarTaq *Plus* DNA Polymerase is ideally suited for a wide variety of applications, including all standard PCR applications and challenging applications such as single-cell PCR, nested PCR, or typing studies.

For high-fidelity hot-start PCR we recommend the HotStar HiFidelity Polymerase Kit for highly sensitive and reliable high-fidelity PCR without optimization.

HotStarTaq *Plus* Procedure



Reaction setup
at room temperature



Distribute



Add primers
and template

Amplification

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

- Reaction tubes
- Pipets and pipet tips (aerosol resistant)
- Thermal cycler
- Mineral oil (only if the thermal cycler does not have a heated lid)
- Primers
 - Primers should be purchased from an established oligonucleotide manufacturer, such as Operon Biotechnologies (www.operon.com). Lyophilized primers should be dissolved in TE 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 .

HotStarTaq *Plus* DNA Polymerase Protocols

- RNase-free water
- dNTPs (e.g., dNTP Mix, PCR Grade, cat. no. 201900)

Protocol: PCR Using HotStarTaq *Plus* DNA Polymerase

Important points before starting

- HotStarTaq *Plus* DNA Polymerase requires an activation step of **5 min at 95°C** (see step 6 of this protocol).
- Set up all reaction mixtures in an area separate from that used for DNA preparation or PCR product analysis.
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- CoralLoad PCR Buffer 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).

Things to do before starting

- If required, prepare a dNTP mix containing 10 mM of each dNTP. Store this mix in aliquots at -20°C. High-quality, PCR-grade dNTP mix (10 mM) is available from QIAGEN (cat. no. 201900).

Procedure

1. **Thaw 10x CoralLoad PCR Buffer or 10x PCR Buffer, dNTP mix, primer solutions, and 25 mM MgCl₂ (if required) at room temperature or on ice.**

It is important to mix the solutions completely before use to avoid localized concentrations of salts.

2. **Prepare a master mix according to Table 1.**

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

The master mix typically contains all the components needed for PCR except the template DNA. Prepare a volume of master mix 10% greater than that required for the total number of PCR assays to be performed. A negative control (without template DNA) should always be included.

Note: The Mg²⁺ concentration provided by the supplied PCR or CoralLoad PCR Buffer will produce satisfactory results in most cases. However, in some cases, reactions may be improved by increasing the final Mg²⁺ concentration according to Table 2.

Table 1. Reaction Composition Using HotStarTaq *Plus* DNA Polymerase

Component	Volume/reaction	Final Concentration
Master mix		
10x CoralLoad PCR Buffer* or 10x PCR Buffer*	10 μ l	1x
25 mM MgCl ₂	Variable, see Table 2	See Table 2
dNTP mix (10 mM of each)	2 μ l	200 μ M of each dNTP
Primer A	Variable	0.1–0.5 μ M [†]
Primer B	Variable	0.1–0.5 μ M [†]
HotStarTaq <i>Plus</i> DNA Polymerase	0.5 μ l	2.5 units/reaction
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	\leq 1 μ g/reaction
Total volume	100 μl	–

Note: If smaller reaction volumes are used, reduce the amount of each component accordingly.

* Contains 15 mM MgCl₂.

[†] See Table 16 (page 36) for molar conversion ratios.

Table 2. Final Mg²⁺ Concentrations

Final Mg ²⁺ concentration in reaction (mM):	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Required volume of 25 mM MgCl ₂ per reaction (μ l):	0	2	4	6	8	10	12	14

Note: The optimal Mg²⁺ concentration should be determined empirically but in most cases a concentration of 1.5 mM, as provided in the 1x PCR Buffer and 1x CoralLoad PCR Buffer, will produce satisfactory results.

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

Mix gently (e.g., by pipetting the master mix up and down a few times). It is not necessary to keep PCR tubes on ice as nonspecific DNA synthesis cannot occur at room temperature due to the inactive state of HotStarTaq Plus DNA Polymerase.

4. Add template DNA (<1 µg/reaction) to the individual tubes containing the master mix.

For RT-PCR, add an aliquot from the reverse transcriptase reaction. This should not exceed 10% of the final PCR volume (see Appendix E, page 40).

5. When using a thermal cycler with a heated lid, do not use mineral oil. Proceed directly to step 6. Otherwise, overlay with approximately 100 µl mineral oil.**6. Program the thermal cycler according to the manufacturer's instructions.**

Note: Each PCR program must start with an initial heat activation step at 95°C for 5 min. Do not exceed the 5 min activation time.

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

Table 3. 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:	0.5–1 min	94°C	
Annealing:	0.5–1 min	50–68°C	Approximately 5°C below T_m of primers (see Appendix B, page 36).
Extension:	1 min	72°C	For PCR products longer than 1 kb, use an extension time of approximately 1 min/kb DNA.
Number of cycles:	25–35		See Appendix C, page 39.
Final extension:	10 min	72°C	

7. 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.

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

CoralLoad PCR Buffer contains a gel loading reagent and gel tracking dyes. Refer to Table 4 below 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 4. Migration Distance of Gel Tracking Dyes

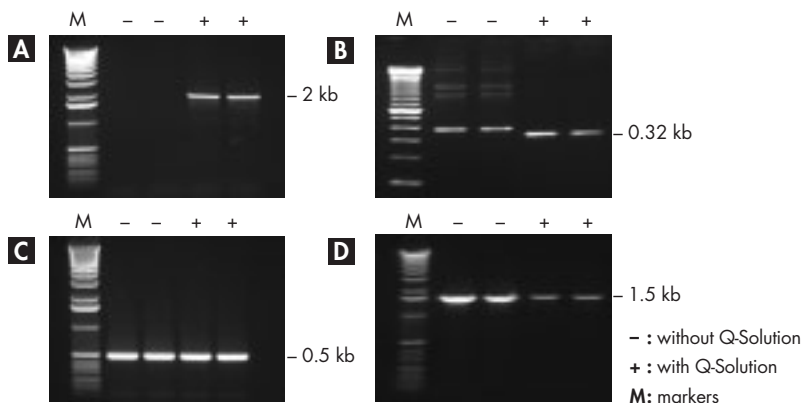
%TAE (TBE) agarose gel	Red dye	Orange dye
0.8	500 bp (270 bp)	~80 bp (<10 bp)
1.0	300 bp (220 bp)	~40 bp (<10 bp)
1.5	250 bp (120 bp)	~20 bp (<10 bp)
2.0	100 bp (110 bp)	<10 bp (<10 bp)
3.0	50 bp (100 bp)	<10 bp (<10 bp)

Protocol: PCR Using HotStarTaq *Plus* 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

- HotStarTaq *Plus* DNA Polymerase requires an activation step of **5 min at 95°C** (see step 6 of this protocol).
- 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.
- Set up all reaction mixtures in an area separate from that used for DNA preparation or PCR product analysis.
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- CoralLoad PCR Buffer 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).

Things to do before starting

- If required, prepare a dNTP mix containing 10 mM of each dNTP. Store this mix in aliquots at –20°C. High-quality, PCR-grade dNTP mix (10 mM) is available from QIAGEN (cat. no. 201900).

Procedure

- 1. Thaw 10x CoralLoad PCR Buffer or 10x PCR Buffer, dNTP mix, primer solutions, and Q-Solution at room temperature or on ice.**

It is important to mix the solutions completely before use to avoid localized concentrations of salts. When using Q-Solution, additional $MgCl_2$ is not usually required.

- 2. Prepare a master mix at room temperature or on ice according to Table 5.**

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

The master mix typically contains all the components needed for PCR except the template DNA. Prepare a volume of master mix 10% greater than that required for the total number of PCR assays to be performed. A negative control (without template DNA) should always be included.

Table 5. Reaction Composition Using HotStarTaq *Plus* DNA Polymerase and Q-Solution

Component	Volume/reaction	Final Concentration
Master mix		
10x CoralLoad PCR Buffer* or 10x PCR Buffer*	10 μ l	1x
5x Q-Solution	20 μ l	1x
dNTP mix (10 mM of each)	2 μ l	200 μ M of each dNTP
Primer A	Variable	0.1–0.5 μ M [†]
Primer B	Variable	0.1–0.5 μ M [†]
HotStarTaq <i>Plus</i> DNA Polymerase	0.5 μ l	2.5 units/reaction
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	\leq 1 μ g/reaction
Total volume	100 μl	–

Note: If smaller reaction volumes are used, reduce the amount each component accordingly.

* Contains 15 mM MgCl₂.

[†] See Table 16 (page 36) for molar conversion ratios.

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

Mix gently (e.g., by pipetting the master mix up and down a few times). It is not necessary to keep the PCR tubes on ice as nonspecific DNA synthesis cannot occur at room temperature due to the inactive state of HotStarTaq *Plus* DNA Polymerase.

4. Add template DNA (<1 μ g/reaction) to the individual tubes containing the master mix.

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 40).

5. When using a thermal cycler with a heated lid, do not use mineral oil. Proceed directly to step 6. Otherwise, overlay with approximately 100 μ l mineral oil.
6. Program the thermal cycler according to the manufacturer's instructions.

Note: Each PCR program must start with an initial heat activation step at 95°C for 5 min. Do not exceed the 5 min activation time.

A typical PCR cycling program is outlined in Table 6. For maximum yield and specificity, temperatures and cycling times should be optimized for each new template target or primer pair.

Table 6. Optimized Cycling Protocol

			Additional comments
Initial activation step:	5 min	95°C	HotStarTaq <i>Plus</i> DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation:	0.5–1 min	94°C	
Annealing:	0.5–1 min	50–68°C	Approximately 5°C below T_m of primers (see Appendix B, page 36).
Extension:	1 min	72°C	For PCR products longer than 1 kb, use an extension time of approximately 1 min/kb DNA.
Number of cycles:	25–35		See Appendix C, page 39.
Final extension:	10 min	72°C	

7. 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.

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

CoralLoad PCR Buffer contains a gel loading reagent and gel tracking dyes. Please refer to Table 7 below 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 7. 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: Single-Cell PCR Using HotStarTaq *Plus* DNA Polymerase

This protocol is designed for successful amplification of single-copy DNA sequences from single cells.

Important points before starting

- HotStarTaq *Plus* DNA Polymerase requires an activation step of **5 min at 95°C** (see step 5 of this protocol).
- Addition of **carrier nucleic acid** is usually required (e.g., *E. coli* 5S rRNA).
- Use polyacrylamide gel- or HPLC-purified primers only.
- Set up all reaction mixtures in an area separate from that used for DNA preparation or PCR product analysis.
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- CoralLoad PCR Buffer 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).

Important points for cell handling

- Single cells may be isolated by different methods (e.g., by flow cytometry or by micromanipulation).
- Keep samples cool during the cell-isolation procedure to prevent DNA degradation.
- Transfer single cell into a PCR tube that has been filled with 20 μ l of 1x PCR Buffer. Immediately freeze the sample on dry ice.
- Store cell at -80°C until required for PCR analysis.

Things to do before starting

- If required, prepare a dNTP mix containing 10 mM of each dNTP. Store this mix in aliquots at -20°C . High-quality, PCR-grade dNTP mix (10 mM) is available from QIAGEN (cat. no. 201900).

Procedure

1. Thaw 10x CoralLoad PCR Buffer or 10x PCR Buffer, dNTP mix, primer solutions, and 25 mM MgCl₂ (if required) at room temperature or on ice. Thaw cells in PCR tubes on ice.

It is important to mix the solutions completely before use to avoid localized concentrations of salts.

2. Prepare a master mix according to Table 8.

The master mix typically contains all the components needed for PCR except the template DNA (single cell). Prepare a volume of master mix 10% greater than that required for the total number of PCR assays to be performed. A negative control (without template DNA) should always be included.

Note: The Mg²⁺ concentration provided by the supplied PCR or CoralLoad PCR Buffer will produce satisfactory results in most cases. However, in some cases, reactions may be improved by increasing the final Mg²⁺ concentration according to Table 9.

Table 8. Reaction Composition Using HotStarTaq *Plus* DNA Polymerase

Component	Volume/reaction	Final Concentration
Master mix		
10x CoralLoad PCR Buffer* or 10x PCR Buffer*	5 µl	1x
25 mM MgCl ₂	Variable, see Table 9	See Table 9
dNTP mix (10 mM of each)	1 µl	200 µM of each dNTP
Primer A	Variable	0.2 µM [†]
Primer B	Variable	0.2 µM [†]
Carrier RNA	Variable	(50 ng per reaction)
HotStarTaq <i>Plus</i> DNA Polymerase	1 µl	5 units/reaction
RNase-free water	Variable	–
Template		
Single cell in 1x PCR Buffer 20 µl		
Total volume	50 µl	–

* Contains 15 mM MgCl₂.

[†] See Table 16 (page 36) for molar conversion ratios.

Table 9. Final Mg²⁺ Concentrations

Final Mg ²⁺ concentration in reaction (mM):	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Required volume of 25 mM MgCl ₂ per reaction (μl):	0	2	4	6	8	10	12	14

Note: The optimal Mg²⁺ concentration should be determined empirically but in most cases a concentration of 1.5 mM, as provided in the 1x PCR Buffer and 1x CoralLoad PCR Buffer, will produce satisfactory results.

3. Mix the master mix thoroughly and dispense 30 μl into PCR tubes.

Mix gently (e.g., by pipetting the master mix up and down a few times).

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

5. Program the thermal cycler according to the manufacturer's instructions.

Note: Each PCR program must start with an initial heat activation step at 95°C for 5 min. Do not exceed the 5 minute activation time.

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

Table 10. Optimized Cycling Protocol

			Additional comments
Initial activation step:	5 min	95°C	HotStarTaq <i>Plus</i> DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation:	0.5–1 min	94°C	
Annealing:	0.5–1 min	50–68°C	Approximately 5°C below T_m of primers (see Appendix B, page 36).
Extension:	1 min	72°C	For PCR products longer than 1 kb, use an extension time of approximately 1 min/kb DNA.
Number of cycles:	45–50		See Appendix C, page 39.
Final extension:	10 min	72°C	

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 PCR Buffer, the PCR products can be directly loaded onto an agarose gel without prior addition of a PCR loading buffer and gel tracking dyes.

CoralLoad PCR Buffer contains a gel loading reagent and gel tracking dyes. Refer to Table 11 below 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 11. 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 HotStarTaq Plus Master Mix

Important points before starting

- HotStarTaq *Plus* Master Mix requires an activation step of **5 min at 95°C** (see step 6 of this protocol).
- HotStarTaq *Plus* Master Mix provides a final concentration of 1.5 mM MgCl₂ in the final reaction mix, which will produce satisfactory results in most cases. However, if a higher Mg²⁺ concentration is required, prepare a stock solution containing 25 mM MgCl₂.
- CoralLoad Concentrate 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).
- Set up all reaction mixtures in an area separate from that used for DNA preparation or PCR product analysis.
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.

Procedure

1. Thaw primer solutions and template nucleic acid.

Mix well before use.

2. Mix the HotStarTaq *Plus* Master Mix by vortexing briefly and dispense 10 µl into each PCR tube according to Table 12.

It is important to mix the HotStarTaq *Plus* Master Mix before use in order 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 (<200 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 40).

Table 12. Reaction Composition Using HotStarTaq *Plus* Master Mix

Component	Volume/reaction	Final Concentration
HotStarTaq <i>Plus</i> Master Mix, 2x	10 μ l	1 unit HotStarTaq <i>Plus</i> DNA Polymerase 1 x PCR Buffer* 200 μ M of each dNTP
Diluted primer mix		
Primer A	Variable	0.1–0.5 μ M
Primer B	Variable	0.1–0.5 μ M
Optional: CoralLoad Concentrate, 10x	2 μ l	1x CoralLoad Concentrate
RNase-Free Water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	<200 ng/20 μ l reaction
Total volume	20 μl	–

Note: If smaller or larger reaction volumes are used, adjust the amount of each component accordingly.

* Contains 1.5 mM MgCl₂.

- When using a thermal cycler with a heated lid, do not use mineral oil. Proceed directly to step 6. Otherwise, overlay with approximately 50 μ l mineral oil.
- Program the thermal cycler according to the manufacturer's instructions.

Note: Each PCR program must start with an initial heat activation step at 95°C for 5 min. Do not exceed the 5 min activation time.

A typical PCR cycling program is outlined in Table 13 (page 30). For maximum yield and specificity, temperatures and cycling times should be optimized for each new template target or primer pair.

Table 13. Optimized Cycling Protocol

			Additional comments
Initial activation step:	5 min	95°C	HotStarTaq <i>Plus</i> DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation:	0.5–1 min	94°C	
Annealing:	0.5–1 min	50–68°C	Approximately 5°C below T_m of primers (see Appendix B, page 36).
Extension:	1 min	72°C	For PCR products longer than 1 kb, use an extension time of approximately 1 min per kb DNA.
Number of cycles:	25–35		See Appendix C, page 39.
Final extension:	10 min	72°C	

7. 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.

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

CoralLoad PCR Buffer contains a gel loading reagent and gel tracking dyes. Please refer to Table 14 below 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 14. 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 <i>Plus</i> DNA Polymerase not activated | Check whether PCR was started with an initial incubation step at 95°C for 5 min.
An activation time of 15 min may lead to primer–dimer formation or multibanded products |
| b) | Pipetting error or missing reagent | Repeat the PCR. Check the concentrations and storage conditions of reagents, including primers and dNTP mix. In the case of HotstarTaq <i>Plus</i> Master Mix, ensure a 1:1 ratio of HotStarTaq <i>Plus</i> Master Mix to primer–template solution is maintained. |
| c) | PCR cycling conditions are not optimal | Using the same cycling conditions, repeat the PCR using Q-Solution. Follow the protocol on page 19. |
| d) | 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.* |
| e) | Problems with starting template | Check the concentration, storage conditions, and quality of the starting template (see Appendix A, page 35). If necessary, make new serial dilutions of template nucleic acid from stock solutions. Repeat the PCR using the new dilutions.

For single-cell PCR, perform a second round of PCR using a nested PCR approach (see Appendix D, page 39). |

* 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 1.5–5.0 mM (in 0.5 mM steps) using a 25 mM MgCl ₂ solution (see Table 2, page 16). |
| g) | Enzyme concentration too low | When using HotStarTaq <i>Plus</i> DNA Polymerase, use 2.5 units per 100 µl reaction. If necessary, increase the amount of HotStarTaq <i>Plus</i> DNA Polymerase (in 0.5 unit steps). When using HotStarTaq <i>Plus</i> Master Mix, use 10 µl Master Mix per 20 µl reaction. |
| h) | Insufficient number of cycles | Increase the number of cycles in steps of 5 cycles (see Appendix C, page 39). |
| i) | Incorrect annealing temperature or time | Decrease annealing temperature in 2°C steps. Annealing time should be between 30 and 60 s. Difficulties in determining the optimal annealing temperature can be overcome in many cases by performing touchdown PCR (see Appendix F, page 42). |
| j) | Incorrect denaturation temperature or time | Denaturation should be at 94°C for 30 to 60 s. Ensure that the initial 5 min 95°C incubation step was performed as described in step 6 of the PCR protocols (pages 17 and 22) or step 5 of the single-cell protocol (page 26). |
| k) | Extension time too short | Increase the extension time in increments of 1 min. For PCR using genomic DNA, follow suggestion “o) PCR of long fragments from genomic DNA”, below. |
| l) | Insufficient starting template | Perform a second round of PCR using a nested PCR approach (see Appendix D, page 39). |
| m) | Primer design not optimal | Review primer design (see Appendix B, page 36). |
| n) | 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 40). |

Comments and suggestions

- o) 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 35). Alternatively, use the protocol for amplification of long PCR products using QIAGEN *Taq* DNA Polymerase and ProofStart® DNA Polymerase or HotStar HiFidelity Polymerase (see the *Taq PCR Handbook* or the *HotStar HiFidelity PCR Handbook*).
- p) 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.
- q) 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) 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 19.
- c) Annealing temperature too low
Increase annealing temperature in 2°C steps. Annealing time should be between 30 and 60 s. Difficulties in determining the optimal annealing temperature can be overcome in many cases by performing touchdown PCR (see Appendix F, page 42).
- d) 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.*
- e) Primer design not optimal
Review primer design (see Appendix B, page 36).

* 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.

Product is smeared

- | | | |
|----|--|---|
| a) | Too much starting template | <p>Check the concentration and storage conditions of the starting template (see Appendix A, page 35). Make serial dilutions of template nucleic acid from stock solutions.</p> <p>Perform PCR using these serial dilutions. When re-amplifying a PCR product, start the re-amplification round using 1 μl of a 1-in-10^3 – 10^4 dilution of the previous PCR. In most cases, a nested PCR approach results in higher specificity and sensitivity for reamplification (see Appendix D, page 39).</p> |
| b) | Carryover contamination | <p>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 that used for DNA preparation or PCR product analysis.</p> |
| c) | Enzyme concentration too high | <p>When using HotStarTaq <i>Plus</i> DNA Polymerase, use 2.5 units per 100 μl reaction. When using HotStarTaq <i>Plus</i> Master Mix, use 10 μl Master Mix per 20 μl reaction.</p> |
| d) | HotStarTaq <i>Plus</i> DNA Polymerase activation time too long | <p>Check whether PCR was started with an initial incubation step at 95°C for only 5 min.</p> |
| e) | Too many cycles | <p>Reduce the number of cycles in steps of 3 cycles.</p> |
| f) | Mg^{2+} concentration not optimal | <p>Perform PCR with different final concentrations of Mg^{2+} from 1.5–5.0 mM (in 0.5 mM steps) using the 25 mM MgCl_2 solution provided (see Table 2, page 16).</p> |
| g) | Primer concentration not optimal or primers degraded | <p>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.</p> |
| h) | Primer design not optimal | <p>Review primer design (see Appendix B, page 36).</p> |

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, 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).

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 15 and 16 respectively.

Table 15. Spectrophotometric Conversions for Nucleic Acid Templates

1 A₂₆₀ 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 16. Molar Conversions for Nucleic Acid Templates

Nucleic acid	Size	pmol/ μ g	Molecules/ μ g
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			
<i>Escherichia coli</i>	4.7×10^6 *	3.0×10^{-4}	$1.8 \times 10^{8\dagger}$
<i>Drosophila melanogaster</i>	1.4×10^8 *	1.1×10^{-5}	$6.6 \times 10^{5\dagger}$
<i>Mus musculus</i> (mouse)	2.7×10^9 *	5.7×10^{-7}	$3.4 \times 10^{5\dagger}$
<i>Homo sapiens</i> (human)	3.3×10^9 *	4.7×10^{-7}	$2.8 \times 10^{5\dagger}$

* 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 17.

* 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 17. General Guidelines for Standard PCR Primers

Length:	18–30 nucleotides															
G/C content:	40–60%															
T_m:	<p>Simplified formula for estimating melting temperature (T_m): $T_m = 2^\circ\text{C} \times (\text{A}+\text{T}) + 4^\circ\text{C} \times (\text{G}+\text{C})$</p> <p>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.</p>															
Sequence:	<ul style="list-style-type: none"> ■ 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:	<ul style="list-style-type: none"> ■ Spectrophotometric conversion for primers: $1 A_{260} \text{ unit} = 20\text{--}30 \mu\text{g/ml}$ ■ Molar conversions: <table border="1" style="margin-left: 20px;"> <thead> <tr> <th>Primer length</th> <th>pmol/μg</th> <th>20 pmol</th> </tr> </thead> <tbody> <tr> <td>18mer</td> <td>168</td> <td>119 ng</td> </tr> <tr> <td>20mer</td> <td>152</td> <td>132 ng</td> </tr> <tr> <td>25mer</td> <td>121</td> <td>165 ng</td> </tr> <tr> <td>30mer</td> <td>101</td> <td>198 ng</td> </tr> </tbody> </table> <ul style="list-style-type: none"> ■ Use 0.1–0.5 μM of each primer in PCR. For most applications, a primer concentration of 0.2 μM will be sufficient. 	Primer length	pmol/ μg	20 pmol	18mer	168	119 ng	20mer	152	132 ng	25mer	121	165 ng	30mer	101	198 ng
Primer length	pmol/ μg	20 pmol														
18mer	168	119 ng														
20mer	152	132 ng														
25mer	121	165 ng														
30mer	101	198 ng														
Storage:	<p>Lyophilized primers should be dissolved in a small volume of distilled water or TE 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.</p>															

* 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.

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 *Plus* DNA Polymerase often improves the specificity of PCR amplifications that employ degenerate primers by reducing the formation of nonspecific PCR products and primer-dimers. Table 18 gives recommendations for further optimizing PCR using degenerate primers.

Table 19 shows the codon redundancy of each amino acid.

Table 18. Guidelines for Design and Use of Degenerate Primers

Sequence:	<ul style="list-style-type: none">■ Avoid degeneracy in the 3 nucleotides at the 3' end.■ 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:	<ul style="list-style-type: none">■ Begin PCR with a primer concentration of 0.2 μM.■ In case of poor PCR efficiency, increase primer concentrations in increments of 0.25 μM until satisfactory results are obtained.

Table 19. 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

A cycling program usually consists of between 25 and 35 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 20 provides a general guideline for choosing the number of cycles.

Table 20. General Guidelines for Choosing the Number of PCR Cycles

Number of copies of starting template*	1 kb DNA	<i>E. coli</i> 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
$1 \times 10^3 - 5 \times 10^4$	1.1–55 fg	5.56–278 pg	3.6–179 ng	30–35
$>5 \times 10^4$	>55 fg	>278 pg	>179 ng	25–35

* Refer to Table 12 (page 32) 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: Challenging 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 PCR Buffer increases specificity both at the start of and during PCR. Thus HotStarTaq *Plus* DNA Polymerase is well suited to such challenging and highly sensitive PCR assays.

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 HotStarTaq *Plus* DNA Polymerase. Subsequently, an aliquot of the first-round PCR product, for example 1 μ l of a $1\text{-in-}10^3 - 10^4$ 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.

Single-cell PCR

See single-cell PCR protocol on page 24.

Multiplex PCR

Multiplex PCR is a demanding technique that requires extensive optimization of the amounts of DNA polymerase, $MgCl_2$, additional reagents, and primers. Often, cycling parameters need to be changed. In many cases, results are still disappointing and further optimization is required. QIAGEN now offers the QIAGEN Multiplex PCR Kit, which eliminates the need for optimization, making the development of multiplex PCR assays both simple and fast. The QIAGEN Multiplex PCR Kit is the first kit specifically developed for multiplex PCR and provides an easy-to-use master-mix format. QIAGEN Multiplex PCR Master Mix contains pre-optimized concentrations of HotStarTaq DNA Polymerase and $MgCl_2$, plus dNTPs and an innovative PCR buffer specially developed for multiplex PCR. The new PCR buffer contains a balanced combination of salts and additives, which enables comparable efficiencies for annealing and extension of all primers in the reaction. The kit is highly suited for multiplex PCR applications such as typing of genetically modified animals and plants, microsatellite analysis, determination of bacteria and viruses, or amplification of regions carrying SNPs. For more information about the QIAGEN Multiplex PCR Kit, contact your local QIAGEN Technical Services or distributor (see inside back cover).

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. 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 21, page 41.

Table 21. General Guidelines for Performing RT-PCR

<p>RNA purification and reverse transcription:</p>	<p>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 <i>Omniscript Reverse Transcriptase Handbook</i>. Or, when using an enzyme from another supplier, follow the manufacturer's instructions. The following guidelines may be helpful.</p> <ul style="list-style-type: none">■ Mix the following reagents in a microcentrifuge tube:<ul style="list-style-type: none">4.0 µl 5x RT buffer1.0 µl RNase inhibitor (5 units/µl)2.0 µl DTT (0.1 M)1.0 µl each dNTP (10 mM)~1 µg RNA2.5 µl primer (0.2 µg/µl)Reverse Transcriptase† <p>Add RNase-free water to a final volume of 20 µl.</p> <ul style="list-style-type: none">■ Incubate following the manufacturer's instructions.■ Heat the reaction mix at 95°C for 5 min to inactivate the reverse transcriptase.
<p>PCR:</p>	<ul style="list-style-type: none">■ Prepare a PCR mixture following steps 1–3 in the protocols.■ Add 2–5 µl from the RT reaction to each PCR tube containing the master mix.■ Continue with step 4 in the PCR protocols (step 4 of HotStarTaq <i>Plus</i> Master Mix protocol).

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 or visit www.qiagen.com).

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

Appendix F: 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 G: 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 H: 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.
- Prepare and freeze small aliquots of primer solutions and dNTP mix. Use of fresh distilled 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 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 material safety data sheets (MSDSs), available from the product supplier.

Ordering Information

Product	Contents	Cat. no.
HotStarTaq <i>Plus</i> DNA Polymerase (250 U)	250 units HotStarTaq <i>Plus</i> DNA Polymerase, 10x PCR Buffer, * 10x CoralLoad PCR Buffer, * 5x Q-Solution, 25 mM MgCl ₂	203603
HotStarTaq <i>Plus</i> DNA Polymerase (1000 U)	1000 units HotStarTaq <i>Plus</i> DNA Polymerase, 10x PCR Buffer, * 10x CoralLoad PCR Buffer, * 5x Q-Solution, 25 mM MgCl ₂	203605
HotStarTaq <i>Plus</i> DNA Polymerase (5000 U)	5000 units HotStarTaq <i>Plus</i> DNA Polymerase, 10x PCR Buffer, * 10x CoralLoad PCR Buffer, * 5x Q-Solution, 25 mM MgCl ₂	203607
HotStarTaq <i>Plus</i> DNA Polymerase (25000 U)	25000 units HotStarTaq <i>Plus</i> DNA Polymerase, 10x PCR Buffer, * 10x CoralLoad PCR Buffer, * 5x Q-Solution, 25 mM MgCl ₂	203609
HotStarTaq <i>Plus</i> Master Mix Kit (250 U)*†	For 250 x 20 µl reactions: 2x HotStarTaq <i>Plus</i> Master Mix, 10x CoralLoad Concentrate, RNase free water	203643
HotStarTaq DNA Polymerase (250 U)*†	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer, * 5x Q-Solution, 25 mM MgCl ₂	203203
HotStarTaq Master Mix Kit (250 U)*†	3 x 0.85 ml HotStarTaq Master Mix containing 250 units HotStarTaq DNA Polymerase total, 2 x 1.7 ml distilled water	203443
dNTP Mix, PCR Grade (200 µl)	Mix containing 10 mM each of dATP, dCTP, dGTP, and dTTP (1 x 200 µl)	201900
dNTP Mix, PCR Grade (800 µl)	Mix containing 10 mM each of dATP, dCTP, dGTP, and dTTP (4 x 200 µl)	201901

Related products

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 Q-Solution, 25 mM MgCl ₂	200203
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* Contains 15 mM MgCl₂.

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

Ordering Information

Product	Contents	Cat. no.
TopTaq DNA Polymerase (1000)	1000 units TopTaq DNA Polymerase, 10x PCR Buffer*, CoralLoad Concentrate, 5x Q-Solution, 25 mM MgCl ₂	200205
HotStar HiFidelity Polymerase Kit — For highly sensitive and reliable high-fidelity hot-start PCR		
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
Taq DNA Polymerase — For standard and specialized PCR applications		
Taq DNA Polymerase (250 U) [‡]	250 units Taq DNA Polymerase, 10x PCR Buffer,* 10x CoralLoad PCR Buffer,* 5x Q-Solution, 25 mM MgCl ₂	201203
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

* Contains 15 mM MgCl₂.

[†] Contains Factor SB, dNTPs, and optimized concentration of MgSO₄.

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

Ordering Information

Product	Contents	Cat. no.
QIAxcel DNA Large Fragment Kit (600)	QIAxcel DNA Large Fragment Gel Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips	929006
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
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
QIAquick Gel Extraction Kit—For gel extraction or cleanup of DNA (70 bp to 10 kb) from enzymatic reactions.		
QIAquick Gel Extraction Kit (50)*	50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28704
DNeasy 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

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