

HotStarTaq[®] PCR Handbook

For
HotStarTaq DNA Polymerase
HotStarTaq Master Mix Kit

November 2002



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Kit Contents

HotStarTaq® DNA Polymerase	(250 units)	(1000 units)
Catalog no.	203203	203205
HotStarTaq DNA Polymerase	250 units	4 x 250 units
PCR Buffer, 10x*	1.2 ml	4 x 1.2 ml
Q-Solution, 5x	2 ml	4 x 2 ml
MgCl ₂ , 25 mM	1.2 ml	4 x 1.2 ml
Handbook	1	1

HotStarTaq Master Mix Kit	(250 units)	(1000 units)
Catalog no.	203443	203445
HotStarTaq Master Mix†	3 x 0.85 ml	12 x 0.85 ml
Distilled water	2 x 1.7 ml	8 x 1.7 ml
Handbook	1	1

* Contains 15 mM MgCl₂

† Contains HotStarTaq DNA Polymerase, PCR Buffer with 3 mM MgCl₂, and 400 μM each dNTP

Shipping Conditions

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

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

Storage and Stability

HotStarTaq DNA Polymerase and HotStarTaq Master Mix 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, these products can be kept at least until the expiration date (see the inside of the kit lid) without showing any reduction in performance. HotStarTaq Master Mix Kit can also be stored at 2–8°C for up to 2 months.

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 DNA Polymerase, HotStarTaq Master Mix, 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 call one of the QIAGEN Technical Service Departments or local distributors listed on the last page.

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

Introduction

HotStarTaq DNA Polymerase has been developed by QIAGEN to provide hot-start PCR for higher PCR specificity. The combination of HotStarTaq DNA Polymerase and the unique QIAGEN 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 DNA Polymerase makes hot-start PCR simple and easy, eliminating the extra handling steps and contamination risks associated with conventional hot-start methods.

HotStarTaq DNA Polymerase

HotStarTaq DNA Polymerase is a modified form of the recombinant 94 kDa *Taq* DNA Polymerase from QIAGEN. HotStarTaq DNA Polymerase is provided in an inactive state with no polymerase activity at ambient temperatures. This prevents the formation of misprimed products and primer–dimers at low temperatures. HotStarTaq DNA Polymerase is activated by a 15-minute, 95°C incubation step, which can easily be incorporated into existing thermal cycling programs. HotStarTaq 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.

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

The HotStarTaq DNA 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. For further information, please read the PCR Protocol Using HotStarTaq DNA Polymerase and Q-Solution, page 12.

* For further information see our guide Critical Factors for Successful 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 inside front cover).

Product Specifications

Enzyme:

HotStarTaq 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).

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

Concentration:	5 units/ μ l
Substrate analogs:	dNTP, ddNTP, dUTP, biotin-11-dUTP, DIG-11-dUTP, 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
Protease 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).
Q-Solution:	5x concentrated
MgCl_2 solution:	25 mM

HotStarTaq Master Mix: 2x concentrated. Contains HotStarTaq DNA Polymerase, PCR Buffer (with 3 mM MgCl_2), and 400 μ M each dNTP.

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 HotStarTaq DNA Polymerase in assay buffer (25 mM TAPS [tris-(hydroxymethyl)-methyl-amino-propane-sulfonic acid, sodium salt], pH 9.3 at 20°C; 50 mM KCl; 2 mM MgCl ₂ ; 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. HotStarTaq DNA Polymerase is activated by heating for 3 hours at 80°C prior to activity measurement.
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 DNA Polymerase in PCR Buffer. Exonuclease activity per unit of enzyme is indicated under "Exo".
Endonuclease activity assay:	Plasmid DNA is incubated with HotStarTaq DNA Polymerase in PCR Buffer. Endonuclease activity per unit of enzyme is indicated under "Endo".
RNase activity assay:	RNA is incubated with HotStarTaq DNA Polymerase in PCR Buffer. RNase activity per unit of enzyme is indicated under "RNase".
Protease activity assay:	HotStarTaq DNA Polymerase is incubated in storage buffer. Protease activity per unit of enzyme is indicated under "Protease".
Self-priming activity assay:	Assays are performed under standard PCR conditions, without primers, using HotStarTaq 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, sterility, and performance in PCR are tested.
Q-Solution, 5x:	Conductivity, pH, sterility, and performance in PCR are tested.
MgCl ₂ , 25 mM:	Conductivity, pH, sterility, and performance in PCR are tested.
Distilled water:	Conductivity, pH, sterility, and performance in PCR are tested. Endonuclease, exonuclease, and RNase activities are tested.

HotStarTaq Master Mix Kit:

PCR reproducibility assay:	The PCR reproducibility assay described above is performed in parallel using HotStarTaq Master Mix and using the separate reagents with the same lot numbers.
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PCR Protocol Using HotStarTaq DNA Polymerase

This protocol serves only as a guideline for PCR amplification. Optimal reaction conditions such as incubation times, temperatures, and amount of template DNA may vary and must be individually determined.

Notes: • **HotStarTaq DNA Polymerase requires an activation step of 15 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.
- If required, prepare a dNTP mix containing 10 mM of each dNTP. Store this mix in aliquots at -20°C .

1. Thaw 10x PCR Buffer, dNTP mix, primer solutions, and 25 mM MgCl₂ (if required).

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.

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. 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, will produce satisfactory results.

Table 1. Reaction composition using HotstarTaq DNA Polymerase

Component	Volume/reaction	Final concentration
Master mix		
10x PCR Buffer*	10 μl	1x
25 mM MgCl ₂	Variable, see Table 2	See Table 2
dNTP mix (10 mM of each)	2 μl	200 μM of each dNTP
Primer A	Variable	0.1–0.5 μM
Primer B	Variable	0.1–0.5 μM
HotStarTaq DNA Polymerase	0.5 μl	2.5 units/reaction
Distilled water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	≤ 1 μg /reaction
Total volume	100 μl	–

* Contains 15 mM MgCl₂

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 (μl):	0	2	4	6	8	10	12	14

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 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, page 28).

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.**

Each PCR program must start with an initial heat activation step at 95°C for 15 min. 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.

			Additional comments
Initial activation step:	15 min	95°C	HotStarTaq 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, page 24).
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, page 25.
Final extension:	10 min	72°C	

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

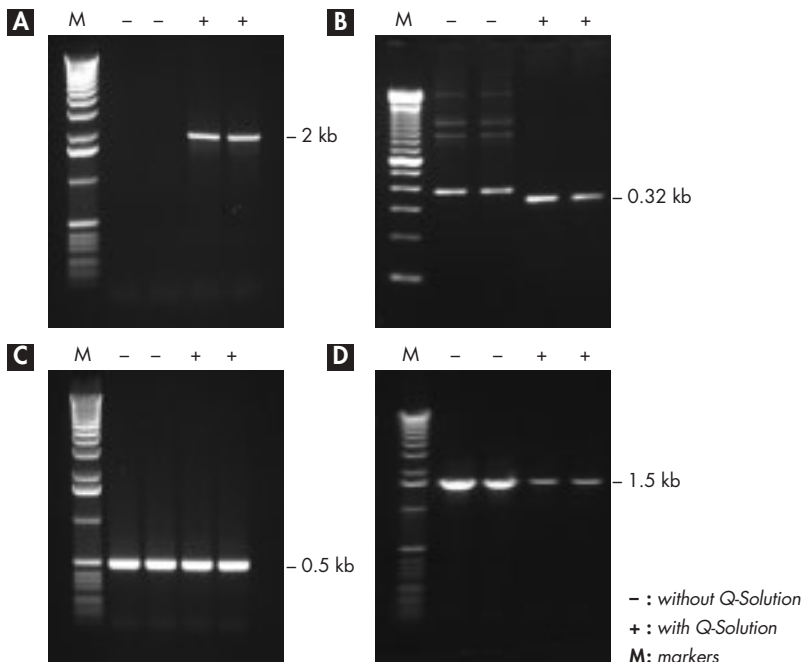
Note: After amplification, samples can be stored overnight at 2–8°C or at –20°C for longer storage.

PCR Protocol Using HotStarTaq 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.



- Notes:**
- HotStarTaq DNA Polymerase requires an activation step of 15 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.
 - If required, prepare a dNTP mix containing 10 mM of each dNTP. Store this mix in aliquots at –20°C.

1. Thaw 10x PCR Buffer, dNTP mix, primer solutions, and Q-Solution.

It is important to mix the solutions completely before use to avoid localized concentrations of salts. When using Q-Solution, additional MgCl₂ is not usually required.

2. Prepare a master mix according to Table 3.

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 3. Reaction composition using HotStarTaq DNA polymerase and Q-Solution

Component	Volume/reaction	Final concentration
Master mix		
10x PCR Buffer*	10 µl	1x
5x Q-Solution	20 µl	1x
dNTP mix (10 mM of each)	2 µl	200 µM of each dNTP
Primer A	Variable	0.1–0.5 µM
Primer B	Variable	0.1–0.5 µM
HotStarTaq DNA Polymerase	0.5 µl	2.5 units/reaction
Distilled water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	≤1 µg/reaction
Total volume	100 µl	–

* Contains 15 mM MgCl₂

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 DNA Polymerase.

4. Add template DNA ($\leq 1 \mu\text{g}/\text{reaction}$) 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, page 28).

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.**

Each PCR program must start with an initial heat activation step at 95°C for 15 min. A typical PCR cycling program is outlined on the next page. For maximum yield and specificity, temperatures and cycling times should be optimized for each new template target or primer pair.

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.

			Additional comments
Initial activation step:	15 min	95°C	HotStarTaq 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, page 24).
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, page 25.
Final extension:	10 min	72°C	

PCR Protocol Using HotStarTaq Master Mix

This protocol serves only as a guideline for PCR amplification. Optimal reaction conditions, such as incubation times and temperatures, and amount of template DNA, may vary and need to be determined individually.

Notes: • Each PCR program should be started with an initial activation step of 15 min at 95°C to activate HotStarTaq DNA Polymerase (see step 6 of this protocol).

- HotStarTaq 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.
- Set up 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.

1. Thaw primer solutions.

Mix well before use.

Optional: prepare a primer mix of an appropriate concentration (see Table 4) using the water provided.

This is recommended if several amplification reactions using the same primer pair are to be performed. The final volume of diluted primer mix should be 25 µl per reaction including the template DNA, added at step 4.

2. Mix the HotStarTaq Master Mix by vortexing briefly and dispense 25 µl into each PCR tube according to Table 4.

It is important to mix the HotStarTaq Master Mix before use in order to avoid localized concentrations of salt. HotStarTaq Master Mix is provided as a 2x concentrate (i.e., a 25 µl volume of the HotStarTaq Master Mix is required for amplification reactions with a final volume of 50 µl). For volumes smaller than 50 µl, the 1:1 ratio of HotStarTaq Master Mix to diluted primer mix and template should be maintained as defined in Table 4. A negative control (without template DNA) should always be included. 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 DNA Polymerase.

3. Distribute the appropriate volume of diluted primer mix into the PCR tubes containing the Master Mix.

4. Add template DNA (≤1 µg/reaction) to the individual PCR tubes.

For RT-PCR, add an aliquot from the reverse transcription reaction. The volume added should not exceed 10% of the final PCR volume (see appendix, page 28).

Table 4. Reaction composition using HotStarTaq Master Mix

Component	Volume/reaction	Final concentration
HotStarTaq Master Mix	25 μ l	2.5 units HotStarTaq DNA Polymerase 1x 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
Distilled water (provided)	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	\leq 1 μ g/reaction
Total volume	50 μl	–

* Contains 1.5 mM MgCl₂

- When using thermal cyclers 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.**
Each PCR program must start with an initial heat activation step at 95°C for 15 min. 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 and primer pair.

			Additional comments
Initial activation step:	15 min	95°C	HotStarTaq 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	5°C below T_m of primers (see appendix, page 24)
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, page 25.
Final extension:	10 min	72°C	

- 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.

Troubleshooting Strategy

This troubleshooting strategy is a process that has been specifically designed to provide a convenient and time-saving optimization procedure for PCR using HotStarTaq DNA Polymerase or the HotStarTaq Master Mix Kit. It is recommended that the troubleshooting steps are followed in the order they are listed. 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 molecular biology applications (see inside front cover for contact information).

Comments and suggestions

Little or no product

- | | |
|---|---|
| 1. HotStarTaq DNA Polymerase not activated | Check whether PCR was started with an initial incubation step at 95°C for 15 min. |
| 2. 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 Master Mix, ensure that a 1:1 ratio of HotStarTaq Master Mix to primer-template solution is maintained. |
| 3. PCR cycling conditions are not optimal | Using the same cycling conditions, repeat the PCR using Q-Solution. Follow the protocol on page 12. |
| 4. 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. |
| 5. Problems with starting template | Check the concentration, storage conditions, and quality of the starting template (see appendix, page 22). If necessary, make new serial dilutions of template nucleic acid from stock solutions. Repeat the PCR using the new dilutions. |
| 6. 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 11). |

Comments and suggestions

- | | |
|--|--|
| 7. Enzyme concentration too low | When using HotStarTaq DNA Polymerase, use 2.5 units per 100 μ l reaction. If necessary, increase the amount of HotStarTaq DNA Polymerase (in 0.5-unit steps). When using HotStarTaq Master Mix, use 25 μ l Master Mix per 50 μ l reaction. |
| 8. Insufficient number of cycles | Increase the number of cycles in steps of 5 cycles (see appendix, page 25). |
| 9. Incorrect annealing temperature or time | Decrease annealing temperature in 2°C steps. Annealing time should be between 30 and 60 seconds. Difficulties in determining the optimal annealing temperature can be overcome in many cases by performing touchdown PCR (see appendix, page 29). |
| 10. Incorrect denaturation temperature or time | Denaturation should be at 94°C for 30 to 60 seconds. Ensure that the initial 15-minute 95°C incubation step was performed as described in step 6 of the PCR protocols (pages 11, 14, and 17). |
| 11. Extension time too short | Increase the extension time in increments of 1 minute. For PCR using genomic DNA, follow suggestion number 15, below. |
| 12. Insufficient starting template | Perform a second round of PCR using a nested PCR approach (see appendix, page 26). |
| 13. Primer design not optimal | Review primer design (see appendix, page 23). |
| 14. 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, page 28). |

15. 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, page 22). Alternatively, use the protocol for amplification of long PCR products using ProofStart™ DNA Polymerase and QIAGEN Taq DNA Polymerase (see the *Taq PCR Handbook*, March 2002, or the *ProofStart PCR Handbook*, February 2002).
16. 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.
17. 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

1. PCR cycling conditions not optimal
Use the same cycling conditions, repeat the PCR using Q-Solution. Follow the protocol on page 12.
2. Annealing temperature too low
Increase annealing temperature in 2°C steps. Annealing time should be between 30 and 60 seconds. Difficulties in determining the optimal annealing temperature can be overcome in many cases by performing touch-down PCR (see appendix, page 29).
3. 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.
4. Primer design not optimal
Review primer design (see appendix, page 23).

Product is smeared

1. Too much starting template
Check the concentration and storage conditions of the starting template (see appendix, page 22). Make serial dilutions of template nucleic acid from stock solutions. 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, page 26).
2. Carry-over contamination
If the negative-control PCR (without template DNA) shows a PCR product or a smear, exchange all reagents. Use disposable pipet tips containing hydrophobic filters to minimize cross-contamination. Set up all reaction mixtures in an area separate from that used for DNA preparation or PCR product analysis.
3. Enzyme concentration too high
When using HotStarTaq DNA Polymerase, use 2.5 units per 100 μl reaction. When using HotStarTaq Master Mix, use 25 μl Master Mix per 50 μl reaction.
4. Too many cycles
Reduce the number of cycles in steps of 3 cycles.
5. 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 the 25 mM MgCl_2 solution provided (see Table 2, page 11).
6. 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.
7. Primer design not optimal
Review primer design (see appendix, page 23).

Appendix

1. 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 inside front 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 5 and 6 respectively.

Table 5. Spectrophotometric conversions for nucleic acid templates

1 A_{260} unit†	Concentration ($\mu\text{g/ml}$)
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40

† Absorbance at 260 nm = 1

* For further information see our guide Critical Factors for Successful 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 inside front cover).

Table 6. 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}	$1.8 \times 10^{8†}$
<i>Drosophila melanogaster</i>	1.4×10^8 *	1.1×10^{-5}	$6.6 \times 10^{5†}$
<i>Mus musculus</i> (mouse)	2.7×10^9 *	5.7×10^{-7}	$3.4 \times 10^{5†}$
<i>Homo sapiens</i> (human)	3.3×10^9 *	4.7×10^{-7}	$2.8 \times 10^{5†}$

* Base pairs in haploid genome

† For single-copy genes

2. 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 7, page 24.‡

‡ For further information see our guide Critical Factors for Successful 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 inside front cover).

Table 7. General guidelines for standard PCR primers

Length:	18–30 nucleotides																
G/C content:	40–60%																
T_m:	Simplified formula for estimating melting temperature (T_m): $T_m = 2^\circ\text{C} \times (\text{A}+\text{T}) + 4^\circ\text{C} \times (\text{G}+\text{C})$ 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:	<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: 40px;"> <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>		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:	<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. 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.																

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 positions 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 nonspecific PCR products and primer–dimers. Table 8 gives recommendations for further optimizing PCR using degenerate primers. Table 9 shows the codon redundancy of each amino acid.

Table 8. 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 9. 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

3. Number of 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 10 provides a general guideline for choosing the number of cycles.

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

Number of copies of starting template*	1 kb DNA	<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 × 10 ³ – 5 × 10 ⁴	1.1–55 fg	5.56–278 pg	3.6–179 ng	30–35
>5 × 10 ⁴	>55 fg	>278 pg	>179 ng	25–35

*Refer to Table 6 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.

4. Sensitive PCR assays

PCR can be performed to amplify and detect just a single copy of a nucleic acid sequence. However, amplification of such a low number of target sequences is often limited by the generation of nonspecific PCR products and primer–dimers. The combination of HotStarTaq DNA Polymerase and QIAGEN PCR Buffer increases specificity both at the start of and during PCR. Thus HotStarTaq DNA Polymerase is well suited to such 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 DNA Polymerase. Subsequently, an aliquot of the first-round PCR product, for example 1 µl of a 1-in-10³–10⁴ 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

HotStarTaq DNA Polymerase has been shown to successfully amplify a single-copy gene from just a single cell. The recommendations provided in Table 11 are intended to serve as a starting point for performing such a single-cell PCR from genomic template DNA. If the PCR product is undetectable or the product yield is too low, perform a nested PCR.

Table 11. Recommendations for single-cell PCR

Isolation and storage of single cells:	<ul style="list-style-type: none"> • 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 cell into a PCR tube that has been filled with 20 μl of 1x PCR Buffer. Immediately freeze the sample on dry ice. • Store cell at -80°C until required for PCR analysis. 																																	
PCR setup:	<ul style="list-style-type: none"> • Prepare a fresh master mix for single-cell PCR (see below). • Thaw the cells on ice. • Distribute 30 μl of the master mix into each PCR tube, and place the tubes in the thermal cycler. Immediately start the cycling program with a 10 min incubation step at 95°C to activate HotStarTaq DNA Polymerase for single-cell PCR. 50 cycles of PCR may be required to amplify a single-copy gene in one round of PCR. 																																	
Master mix preparation:	<p>Prepare a master mix that has a final volume of 30 μl per PCR, as detailed below.</p> <p>Notes:</p> <ul style="list-style-type: none"> • Addition of carrier nucleic acid is usually required (e.g., <i>E. coli</i> 5S rRNA). • Use polyacrylamide gel- or HPLC-purified primers only. 																																	
	<table border="1"> <thead> <tr> <th>Component</th> <th>Volume/reaction</th> <th>Final concentration</th> </tr> </thead> <tbody> <tr> <td>10x PCR Buffer*</td> <td>3 μl</td> <td>1x</td> </tr> <tr> <td>25 mM MgCl_2</td> <td>Variable</td> <td>–</td> </tr> <tr> <td>10 mM dNTP</td> <td>1 μl</td> <td>200 μM of each dNTP</td> </tr> <tr> <td>Primer A</td> <td>Variable</td> <td>0.2 μM</td> </tr> <tr> <td>Primer B</td> <td>Variable</td> <td>0.2 μM</td> </tr> <tr> <td>5S ribosomal RNA (<i>E. coli</i>)</td> <td>Variable</td> <td>50 ng/reaction</td> </tr> <tr> <td>HotStarTaq DNA Polymerase</td> <td>1 μl</td> <td>5 units/reaction</td> </tr> <tr> <td>Distilled water</td> <td>Variable</td> <td>–</td> </tr> <tr> <td>Single cell in 1x PCR Buffer</td> <td>20 μl</td> <td>–</td> </tr> <tr> <td>Total volume</td> <td>50 μl</td> <td>–</td> </tr> </tbody> </table>	Component	Volume/reaction	Final concentration	10x PCR Buffer*	3 μ l	1x	25 mM MgCl_2	Variable	–	10 mM dNTP	1 μ l	200 μ M of each dNTP	Primer A	Variable	0.2 μ M	Primer B	Variable	0.2 μ M	5S ribosomal RNA (<i>E. coli</i>)	Variable	50 ng/reaction	HotStarTaq DNA Polymerase	1 μ l	5 units/reaction	Distilled water	Variable	–	Single cell in 1x PCR Buffer	20 μl	–	Total volume	50 μl	–
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* Contains 15 mM MgCl_2

Multiplex PCR

Multiplex PCR is a demanding technique that requires extensive optimization of the amounts of *Taq* 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 front cover).

5. 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 12 below.

Table 12. General guidelines for performing RT-PCR

RNA purification and reverse transcription:	<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 to 95°C for 5 min to inactivate the reverse transcriptase.
PCR:	<ul style="list-style-type: none">• Prepare a PCR mixture following steps 1–3 in protocols.• Add 2–5 μl from the RT reaction to each PCR tube containing the master mix.• Continue with step 5 in the PCR protocols.

Oligotex is not available in Japan.

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

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

6. 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.

7. 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. For more information about QIAquick and MinElute products, please call QIAGEN Technical Services or your local distributor (see inside front cover).

8. 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.
- Contamination by PCR product carry-over can be eliminated by using the commercially available uracil-N-glycosylase (UNG). The procedure involves substituting dUTP for dTTP in the PCR setup and treating all PCR mixtures with UNG prior to PCR amplification. As a result, any PCR product containing dUTP carried over from previous rounds of amplification is destroyed by cleavage during the initial incubation step that activates HotStarTaq DNA Polymerase.

** Most commercial bleach solutions are approximately 5.25% sodium hypochlorate. Sodium hypochlorate is an irritant and should be handled with caution.*

- 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.

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HotStarTaq DNA Polymerase and HotStarTaq Master Mix Kit are developed, designed, and sold for research purposes only. They are not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

Ordering Information

Product	Contents	Cat. No.
HotStarTaq DNA Polymerase		
HotStarTaq DNA Polymerase (250 U)	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer (containing 15 mM MgCl ₂), 5x Q-Solution, 25 mM MgCl ₂	203203
HotStarTaq DNA Polymerase (1000 U)*	4 x 250 units HotStarTaq DNA Polymerase, 10x PCR Buffer (containing 15 mM MgCl ₂), 5x Q-Solution, 25 mM MgCl ₂	203205
HotStarTaq Master Mix Kit		
HotStarTaq Master Mix Kit (250 U)	3 x 0.85 ml HotStarTaq Master Mix, containing 250 units HotStarTaq DNA Polymerase total and providing a final concentration of 1.5 mM MgCl ₂ and 200 µM each dNTP; 2 x 1.7 ml distilled water	203443
HotStarTaq Master Mix Kit (1000 U)	12 x 0.85 ml HotStarTaq Master Mix, containing 1000 units HotStarTaq DNA Polymerase total and providing a final concentration of 1.5 mM MgCl ₂ and 200 µM each dNTP; 8 x 1.7 ml distilled water	203445
QIAGEN Multiplex PCR Kit		
QIAGEN Multiplex PCR Kit (100)	For 100 x 50 µl multiplex PCR reactions: 2x QIAGEN Multiplex PCR Master Mix (providing a final concentration of 3 mM MgCl ₂ , 3 x 0.85 ml), 5x Q-Solution (1 x 2.0 ml), distilled water (2 x 1.7 ml)	206143

*Larger quantities available; please inquire

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