October 2010

QIAGEN® Multiplex PCR Handbook

For fast and efficient multiplex PCR without optimization

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

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

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- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
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Kit Contents

QIAGEN® Multiplex PCR Kit Catalog no. No. of 50 µl reactions	206143 100	206145 1000
2x QIAGEN Multiplex PCR Master Mix, containing: HotStarTaq® DNA Polymerase, Multiplex PCR Buffer*, dNTP Mix	3 x 0.85 ml	1 x 25 ml
Q-Solution, 5x	1 x 2.0 ml	1 x 10 ml
RNAse-Free Water	2 x 1.7 ml	1 x 20 ml
Handbook	1	1

* Contains 6 mM $MgCl_2$.

Shipping and Storage Conditions

The QIAGEN Multiplex PCR Kit is shipped on dry ice, but retains full activity at room temperature (15–25°C) for 2 days. The QIAGEN Multiplex PCR Kit, including Q-Solution and RNase-free water, 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. The QIAGEN Multiplex PCR Master Mix can be stored at 2–8°C for up to 6 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 the QIAGEN Multiplex PCR 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).

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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 Use Limitations

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

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.

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 .

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Quality Control

QIAGEN Multiplex PCR Master Mix

Multiplex PCR performance assay	PCR performance, reproducibility, and sensitivity are tested in parallel multiplex reactions.
HotStarTaq DNA Polymerase (included in QIAGEN Multiplex PCR Master Mix)	Amplification efficiency and reproducibility in PCR are tested. Functional absence of exonucleases and endonucleases is tested.

Buffers and reagents

QIAGEN Multiplex PCR Buffer	Density, pH, and content of cationic components are tested.
Q-Solution	Conductivity, density, pH, and performance in PCR are tested.
RNase-Free Water	Conductivity, pH, and RNase activities are tested.

Product Specifications

2x QIAGEN Multiplex PCR Master Mix contains:

HotStarTaq DNA Polymerase	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)
QIAGEN Multiplex PCR Buffer	Contains 6 mM $MgCl_2$, pH 8.7 (20°C)
dNTP Mix	Contains dATP, dCTP, dGTP, dTTP; ultrapure quality
Q-Solution	5x concentrated
RNase-Free Water	Ultrapure quality, PCR-grade

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Introduction

Multiplex PCR is a powerful technique that enables amplification of two or more products in parallel in a single reaction tube. It is widely used in genotyping applications and different areas of DNA testing in research, forensic, and diagnostic laboratories. Multiplex PCR can also be used for qualitative and semi-quantitative gene expression analysis using cDNA as a starting template (Table 1). DNA tested typically originates from a variety of eukaryotic (human, animal, and plant) and prokaryotic (bacterial and viral) sources.

Table 1. Applications of Multiplex PCR

Source of DNA	Application
Animals/human	Analysis of satellite DNA (e.g., short tandem repeat [STR] analysis or variable number of tandem repeats [VNTR] analysis)
	Typing of transgenic animals
	Lineage analysis (e.g., of farm animals)
	Detection of pathogens
	Food analysis
	Sex determination
	Detection of mutations
	Amplification of SNP loci
	Qualitative and semi-quantitative gene expression analysis
Plants	Analysis of satellite DNA (e.g., STR or VNTR analysis)
	Typing of transgenic plants
	Lineage analysis
	GMO analysis
	Detection of pathogens
	Qualitative and semi-quantitative gene expression analysis
Bacteria/viruses	Hygiene analysis
	Detection of pathogens/diagnostics
	Qualitative and semi-quantitative gene expression analysis

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The QIAGEN Multiplex PCR Kit is the first commercially available kit for multiplex PCR. It minimizes the need for optimization, making the development of multiplex PCR assays both simple and fast. The kit contains a master mix whose composition and elements were specifically developed for multiplex PCR applications. The master mix contains pre-optimized concentrations of HotStarTaq DNA Polymerase and $MgCl_2$, plus dNTPs, and a PCR buffer that contains the novel factor MP. Use of a master-mix format reduces time and handling for reaction setup and increases reproducibility by eliminating many possible sources of pipetting errors.

HotStarTaq DNA Polymerase

The QIAGEN Multiplex PCR Master Mix contains HotStarTaq DNA Polymerase, a modified form of QIAGEN *Taq* DNA Polymerase. 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 during reaction setup and the first denaturation step, leading to exceptionally high PCR specificity. HotStarTaq DNA Polymerase is easily activated by a 15-minute, 95°C incubation step, which is easy to incorporate into existing thermal cycling programs. The hot start enables reactions to be set up at room temperature, making setup rapid and convenient.

QIAGEN Multiplex PCR Buffer

The unique QIAGEN Multiplex PCR Buffer facilitates the amplification of multiple PCR products. Lengthy optimization procedures, such as adjusting the amounts of *Taq* DNA polymerase, Mg^{2+}, additional reagents, and primers is virtually eliminated. In contrast to conventional PCR reagents, the QIAGEN Multiplex PCR Buffer contains a balanced combination of salts and additives to ensure comparable efficiencies for annealing and extension of all primers in the reaction. Primer annealing is only marginally influenced by $MgCl_2$ concentration, so optimization by titration of Mg^{2+} is usually not required. The buffer also contains the synthetic factor MP, which allows efficient primer annealing and extension irrespective of primer sequence. Factor MP increases the local concentration of primers at the DNA template and stabilizes specifically bound primers.

Q-Solution

The QIAGEN Multiplex PCR 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 often enables or improves 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, which has been optimized for the requirements of multiplex PCR. It is nontoxic, and does not compromise PCR product purity. For further information, please read "Important Notes", page 14 and the protocol on page 23.

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Important Notes

Primers

The QIAGEN Multiplex PCR Kit can be used with standard-quality primers that can be purchased from established oligonucleotide manufacturers. Primers should be purchased desalted or purified, for example using HPLC, and dissolved in TE* (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) to obtain a 50 or 100 µM stock solution (see Table 2). Primer quality is a crucial factor for successful multiplex PCR. Problems encountered in multiplex PCR are frequently due to the use of incorrect primer concentrations or low-quality primers.

- The functionality and specificity of all primer pairs should be tested in single reactions before combining them in a multiplex PCR assay. General recommendations for multiplex PCR primer design are given in Appendix A, page 33.
- For easy and reproducible handling of the numerous primers used in multiplex PCR, we recommend the preparation of a primer mix containing all primers at equimolar concentrations. As a first step, all primer stock solutions should be normalized to a concentration of 50 µM or 100 µM using TE buffer. For guidelines on preparing a normalized stock solution, see Appendix B, page 34.
- The primer mix should be prepared in TE, as described in Table 2, and stored in small aliquots at –20°C to avoid repeated freezing and thawing. Multiple freeze–thaw cycles of the primer mix may lead to decreased assay performance.

Table 2. Preparation of 10x Primer Mix (Containing Each Primer at 2 µM)*

Concentration of normalized primer stock	50 µM (50 pmol/µl)	100 µM (100 pmol/µl)
Each primer	20 µl	10 µl
TE buffer	Variable	Variable
Total volume	500 µl	500 µl

* Allows preparation of a 10x primer mix containing up to 12 primer pairs (50 µM stock) or containing up to 25 primer pairs (100 µM stock).

Methods of analysis

Primer pairs should be carefully designed. In addition to the sequence of primers, the length of the generated PCR products should also be taken into account. The sizes of the amplicons must differ sufficiently in order to be able to distinguish them from one another using agarose or polyacrylamide gels or capillary electrophoresis systems like the QIAxel system or the Agilent 2100 bioanalyzer system (see Tables 3, 4 and 5). For analysis using high-resolution sequencing instruments, primers must be different sizes or labeled using different fluorescent dyes.

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

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Table 3. Guidelines for Agarose Gel Analysis of Multiplex PCR Products

Minimum difference in size of PCR products	Maximum size of fragments	Concentration of agarose
>200 bp	2000 bp	1.3%
>100–200 bp	1000 bp	1.4–1.6%
>50–100 bp	750 bp	1.7–2.0%
20–50 bp	500 bp	2.5–3.0%
<20 bp*	250 bp	3.0–4.0%

* Efficient separation of PCR products differing in size by about 20 bp is usually possible using standard molecular-biology–grade agarose. For separation of fragments that differ in size by less than 20 bp, we recommend using high-resolution agarose, for example MetaPhor® agarose (FMC Bioproducts). For more information, visit www.cambrex.com .

Table 4. Analysis of Multiplex PCR Products using QIAxcel cartridges on the QIAxel system

QIAxcel cartridge	Application	Fragment size range	Best resolution
QX DNA High resolution Cartridge*	High-resolution genotyping	15 bp - 5 kb	3-5 bp for fragments 100-500 bp 50 bp for fragments 500 bp - 1 kb 200- 500 bp for fragments 1-5 kb
QX DNA Screening Cartridge	Fast PCR screening	15 bp - 5 kb	20-50 bp for fragments 100-500 bp 50-100 bp for fragments 500 bp - 1 kb 500 bp for fragments 1-5 kb
QX Large DNA Fragment Cartridge	Large and long size range	15 bp - 10 kb	3-5 bp for fragments 100-500 bp 50 bp for fragments 500 bp - 1 kb 100 bp for fragments 1-5 kb 500 bp for fragments 5-10 kb

* QX DNA High resolution Cartridge is the recommended cartridge for analysis of typical multiplex PCR products up to 1.5 kb.

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Table 5. Guidelines for Agilent 2100 Bioanalyzer Analysis of Multiplex PCR Products

DNA LabChip® Kit	Sizing range	Sizing resolution	Sizing accuracy
1000	25–1000 bp	5% from 100–500 bp 10% from 500–1000 bp	10%
7500	100–7500 bp	10% from 100–1500 bp	10%
12000	100–12000 bp	5% from 100–1000 bp	15%

Template DNA

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. This includes the QIAprep® system for rapid plasmid purification, the QIAamp® and DNeasy® systems for rapid purification of human, plant, and animal genomic DNA and bacterial and viral nucleic acids, and the RNeasy® system for RNA preparation from a variety of sources. For more information about QIAprep, QIAamp, DNeasy, the PAXgene™ Blood DNA System, and RNeasy products, contact one of our Technical Service Departments (see back cover) or visit the QIAGEN web site at www.qiagen.com .

* 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).

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Quantity of starting template

The annealing efficiency of primers to the 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 6 and 7, respectively. The QIAGEN Multiplex PCR Kit enables successful amplification using a range of template amounts (Figure 1).

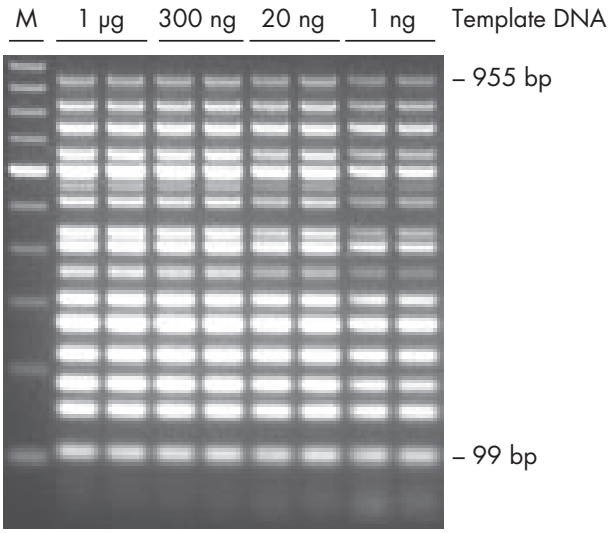

Figure 1. Efficient 16-plex PCR over a Range of Template Amounts. Multiplex PCRs were performed in duplicate using the indicated amount of genomic DNA and 16 pairs of primers that amplified fragments of 99, 150, 181, 222, 269, 310, 363, 414, 446, 523, 564, 610, 662, 756, 845, and 955 bp. **M**: 100 bp ladder.

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Table 6. Spectrophotometric Conversions for Nucleic Acid Templates

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

* Absorbance at 260 nm = 1; 1 cm detection path.

Table 7. Molar Conversions for Nucleic Acid Templates

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

* Base pairs in haploid genome.

† For single-copy genes.

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Choosing the correct protocol

This handbook contains 3 protocols.

Standard Multiplex PCR (page 16)

Choose this protocol for standard multiplex PCR that will be subsequently analyzed using agarose-gel analysis or the Agilent bioanalyzer system.

Amplification of Microsatellite Loci using Multiplex PCR (page 20)

Choose this protocol for amplification of small fragments, for example, analysis of microsatellite loci. Since PCR products generated in these applications are relatively small, the protocol contains a reduced extension time. The inclusion of a final extension step for 30 minutes at 60°C allows generation of A-overhangs by HotStarTaq DNA Polymerase. These overhangs are required for accurate data from high-resolution analysis using capillary- or gel-based DNA sequencers.

Multiplex PCR Using Q-Solution (page 23)

Choose this protocol for PCR systems that are GC rich (≥65% GC content) or have a high degree of secondary structure. It may also be helpful in other cases (see "Multiplex PCR using Q-Solution", page 15).

Special multiplex PCR applications

QIAGEN Multiplex PCR Kit protocols have been developed to give satisfactory results in most cases. In some special cases, modifications to the conditions given in the protocol may improve performance. Such cases include:

- PCR assays with more than 10 products
- PCR of long amplicons (≥1.5 kb)
- Sensitive multiplex PCR assays
- Transgene detection
- SNP analysis
- Detection of genetically modified organisms or microorganisms
- Qualitative or semi-quantitative gene expression analysis
- Exon-specific PCR

For further information on special multiplex PCR applications and optimization recommendations see Appendices E and F on pages 37 and 42, respectively.

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Multiplex PCR using Q-Solution

Q-Solution changes the melting behavior of DNA and will often improve a suboptimal PCR caused by templates that have a high degree of secondary structure or that are GC-rich (≥65% GC content). It may also be helpful in other cases. When using Q-Solution for the first time, 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 system.

We do not recommend combining GC-rich target sequences that either amplify poorly or not at all with target sequences that amplify easily in a multiplex PCR. Nevertheless, many of these primer–template systems can be combined in a multiplex PCR using this protocol.

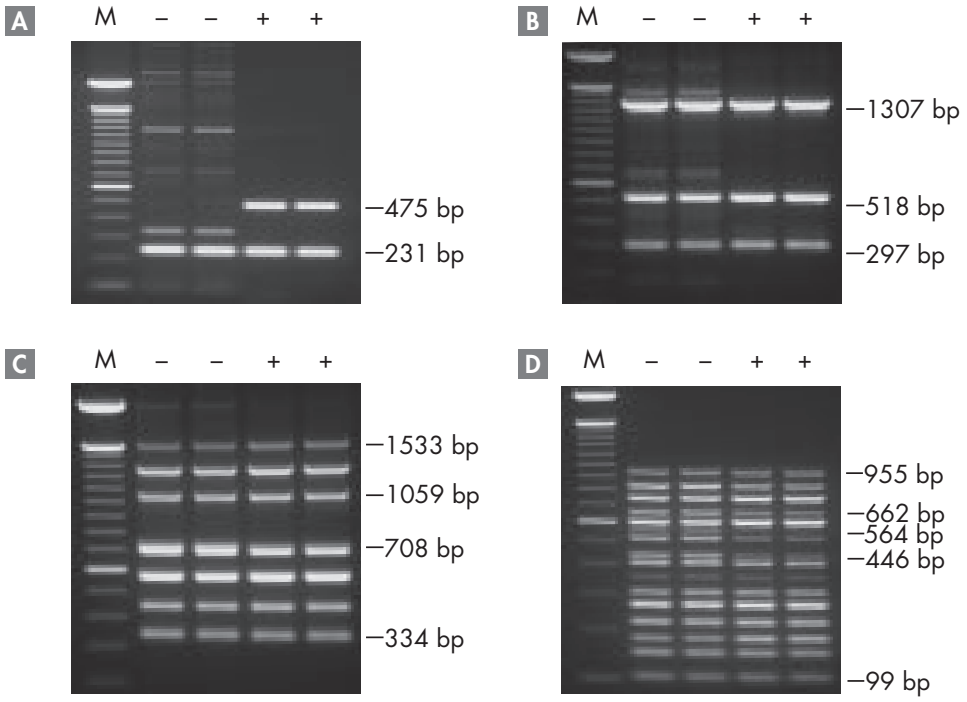
When using Q-Solution, the following effects may be observed depending on the individual PCR assay:

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

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

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

Case D: 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 in a particular multiplex PCR assay, always perform reactions with and without Q-Solution.



– : without Q-Solution; **+ :** with Q-Solution; **M:** markers

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Protocol: Standard Multiplex PCR

This protocol is optimized for all standard multiplex PCR applications. For more advanced applications, such as multiplex reactions with more than 10 products or very low amounts of template, see Appendix F, page 42.

Important notes before starting

- **Always start with the cycling conditions specified in this protocol.**
- If using an already established multiplex PCR system, use the previously established annealing temperature in combination with the cycling conditions specified in this protocol.
- **Annealing time must be 90 seconds.**
- **Use equal concentrations (0.2 µM) of all primers.**
- PCR must start with an **activation step of 15 minutes at 95°C** to activate HotStarTaq DNA Polymerase (see step 7 of this protocol).
- **Optional**: If a thermal cycler with a temperature gradient function can be used, determine the optimal annealing temperature by performing a gradient PCR (see Appendix C, page 36.

Procedure

1. **Thaw 2x QIAGEN Multiplex PCR Master Mix (if stored at –20°C), template DNA, RNase-free water, and primer mix. Mix the solutions completely before use.**

   It is important to mix the solutions completely before use to avoid localized concentrations of salts. Preparing a mixture of all primers avoids pipetting of individual primers for each experiment, reducing pipetting time and increasing reproducibility of results (for preparation of primer mix see Table 2, page 9).

2. **Prepare a reaction mix according to Table 8.**

   The reaction mix typically contains all the components required for multiplex PCR except the template DNA. Prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed. For reaction volumes less than 50 µl, the 1:1 ratio of QIAGEN Multiplex PCR Master Mix to primer mix and template should be maintained as shown in Table 8.

   Note: We strongly recommend starting with an initial Mg^{2+} concentration of 3 mM as provided by the 2x QIAGEN Multiplex PCR Master Mix.

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Table 8. Multiplex PCR Components (Reaction Mix and Template DNA)

Component	Volume/reaction	Final concentration
Reaction mix		
2x QIAGEN Multiplex PCR Master Mix*	25 µl	1x
10x primer mix, 2 µM each primer (see Table 2)	5 µl	0.2 µM†
RNase-free water	Variable	–
Template DNA		
Added at step 4	Variable	≤1 µg DNA/50 µl
Total volume	**50 µl‡**	

* Provides a final concentration of 3 mM $MgCl_2$.

† A final primer concentration of 0.2 µM is optimal for most primer–template systems. However, in some cases using other primer concentrations (0.1–0.3 µM) may further improve amplification performance.

‡ For volumes less than 50 µl, the 1:1 ratio of QIAGEN Multiplex PCR Master Mix to primer mix and template should be maintained.

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

   Mix gently, for example by pipetting the reaction mix up and down a few times. Due to the hot start, it is not necessary to keep samples on ice during reaction setup.

4. **Add template DNA (≤1 µg/50 µl reaction) to the individual PCR tubes containing the reaction mix.**

   For multiplex RT-PCR, the volume of cDNA added (from the RT reaction) as template should not exceed 10% of the final PCR volume.

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

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

   Optional: If a thermal cycler with a temperature gradient function can be used, determine the optimal annealing temperature by performing a gradient PCR.

7. **Place the PCR tubes in the thermal cycler and start the cycling program as outlined in Table 9, page 19.**

   Each PCR program must start with an initial heat-activation step at 95°C for 15 min to activate HotStarTaq DNA Polymerase.

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

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Table 9. Universal Multiplex Cycling Protocol

			Additional comments
Initial activation step:	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:			
Denaturation	30 s	94°C	
Annealing	90 s	57–63°C	**If a gradient PCR cannot be performed, use 60°C as the starting annealing temperature. If the lowest T_m* of your primer mixture is below 60°C, use 57°C as starting annealing temperature.**
Extension	90 s	72°C	Optimal for targets up to approximately 1.5 kb in length.†
Number of cycles	30–45		The number of cycles is dependent on the amount of template DNA and the required sensitivity of your detection method. See Appendix C, page 36 for guidelines.
Final extension:	10 min	72°C	

* T_m determined according to the formula: T_m = 2°C x (number of [A+T]) + 4°C x (number of [G+C]).

† For targets longer than 1.5 kb, an extension time of 2 min may improve performance.

8. **Analyze samples using an appropriate detection system, for example agarose gel electrophoresis (see Table 3, page 10 for choosing the optimal percentage of agarose), polyacrylamide gel electrophoresis*, or capillary electrophoresis.**

   The optimal amount of PCR product required to give a satisfactory signal with your detection method should be determined individually.

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

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Protocol: Amplification of Microsatellite Loci Using Multiplex PCR

This protocol is optimized for standard multiplex PCR amplification of microsatellite loci. For more advanced applications, such as multiplex reactions with more than 10 products or very low amounts of template , see Appendix F, page 42.

Important points before starting

- **Always start with the cycling conditions specified in this protocol.**
- If using an already established multiplex PCR system, use the previously established annealing temperature in combination with the cycling conditions specified in this protocol.
- **Annealing time must be 90 seconds.**
- **Use equal concentrations (0.2 µM) of all primers.**
- PCR must start with an **activation step of 15 minutes at 95°C** to activate HotStarTaq DNA Polymerase (see step 7 of this protocol).
- **Optional**: If a thermal cycler with a temperature gradient function can be used, determine the optimal annealing temperature by performing a gradient PCR (see Appendix C, page 36.

Procedure

1. **Thaw the 2x QIAGEN Multiplex PCR Master Mix (if stored at –20°C), template DNA, RNase-free water, and primer mix. Mix the solutions completely before use.**

   It is important to mix the solutions completely before use to avoid localized concentrations of salts. Preparing a mixture of all primers avoids pipetting of individual primers for each experiment, reducing pipetting time and increasing reproducibility of results (for preparation of primer mix see Table 2, page 9).

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Table 10. Multiplex PCR Components (Reaction Mix and Template DNA)

Component	Volume/reaction	Final concentration
Reaction mix		
2x QIAGEN Multiplex PCR Master Mix*	25 µl	1x
10x primer mix, 2 µM each primer (see Table 2)	5 µl	0.2 µM†
RNase-free water	Variable	–
Template DNA		
Added at step 4	Variable	≤1 µg DNA/50 µl
Total volume	**50 µl‡**	

* Provides a final concentration of 3 mM $MgCl_2$.

† A final primer concentration of 0.2 µM is optimal for most primer–template systems. However, in some cases using other primer concentrations (0.1–0.3 µM) may further improve amplification performance.

‡ For volumes less than 50 µl, the 1:1 ratio of QIAGEN Multiplex PCR Master Mix to primer mix and template should be maintained.

2. **Prepare a reaction mix according to Table 10.**

   The reaction mix typically contains all the components required for multiplex PCR except the template DNA. Prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed. For reaction volumes less than 50 µl, the 1:1 ratio of QIAGEN Multiplex PCR Master Mix to primer mix and template should be maintained as shown in Table 10.

   Note: We strongly recommend starting with an initial Mg^{2+} concentration of 3 mM as provided by the 2x QIAGEN Multiplex PCR Master Mix.

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

   Mix gently, for example by pipetting the reaction mix up and down a few times. Due to the hot start, it is not necessary to keep samples on ice during reaction setup.

4. **Add template DNA (≤1 µg/50 µl reaction) to the individual PCR tubes containing the reaction mix.**

   For multiplex RT-PCR, the volume of cDNA added (from the RT reaction) as template should not exceed 10% of the final PCR volume.

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

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

   Optional: If a thermal cycler with a temperature gradient function can be used, determine the optimal annealing temperature by performing a gradient PCR.

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Table 11. Microsatellite Cycling Protocol

			Additional comments
Initial activation step:	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:			
Denaturation	30 s	94°C	
Annealing	90 s	57–63°C	**If a gradient PCR cannot be performed, use 60°C as the starting annealing temperature. If the lowest T_m* of your primer mixture is below 60°C, use 57°C as starting annealing temperature.**
Extension	60 s	72°C	Optimal for targets up to approximately 0.5 kb in length.†
Number of cycles	25–40		The number of cycles is dependent on the amount of template DNA and the required sensitivity of your detection method. See Appendix C, page 36 for guidelines.
Final extension:	30 min	60°C	

* T_m determined according to the formula: T_m = 2°C x (number of [A+T]) + 4°C x (number of [G+C]).

† For targets longer than 0.5 kb, an extension time of 90 s may improve performance.

7. **Place the PCR tubes in the thermal cycler and start the cycling program as outlined in Table 11.**

   Each PCR program must start with an initial heat-activation step at 95°C for 15 min to activate HotStarTaq DNA Polymerase.

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

8. **Analyze samples using an appropriate detection system, for example automatic gel-based DNA sequencers or those based on capillary electrophoresis.**

   The optimal amount of PCR product required to give a satisfactory signal with your detection method should be determined individually.

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Protocol: Multiplex PCR Using Q-Solution

This protocol is designed for amplification of target sequences that do not work well under standard conditions and has been specifically optimized for multiplex PCR. For more advanced applications, such as multiplex reactions with more than 10 products or very low amounts of template, see Appendix F, page 42. For further information on using Q-Solution, see page 15.

Important points before starting

- **Always start with the cycling conditions specified in this protocol.**
- **When using Q-Solution for the first time in a particular multiplex PCR assay, it is important to perform parallel amplification reactions with and without Q-Solution. Use Q-Solution at a final concentration of 0.5x.**
- If using an already established multiplex PCR system, use the previously established annealing temperature in combination with the cycling conditions specified in this protocol.
- **Annealing time must be 90 seconds.**
- **Use equal concentrations (0.2 µM) of all primers.**
- PCR must start with an **activation step of 15 minutes at 95°C** to activate HotStarTaq DNA Polymerase (see step 7 of this protocol).
- **Optional**: If a thermal cycler with a temperature gradient function can be used, determine the optimal annealing temperature by performing a gradient PCR (see Appendix C, page 36).

Procedure

1. **Thaw the 2x QIAGEN Multiplex PCR Master Mix (if stored at –20°C), template DNA, 5x Q-Solution, RNase-free water, and primer mix. Mix the solutions completely before use.**

   It is important to mix the solutions completely before use to avoid localized concentrations of salts. Preparing a mixture of all primers avoids pipetting of individual primers for each experiment, reducing pipetting time and increasing reproducibility of results (for preparation of primer mix see Table 2, page 9).

2. **Prepare a reaction mix according to Table 12.**

   The reaction mix typically contains all the components required for multiplex PCR except the template DNA. Prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed. For reaction volumes less than 50 µl, the 1:1 ratio of QIAGEN Multiplex PCR Master Mix to primer mix, template, and Q-Solution should be maintained as shown in Table 12.

   Note: We strongly recommend starting with an initial Mg^{2+} concentration of 3 mM as provided by the 2x Multiplex PCR Master Mix.

Q-Solution Protocol

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Table 12. Multiplex PCR Components (Reaction Mix and Template DNA)

Component	Volume/reaction	Final concentration
Reaction mix		
2x QIAGEN Multiplex PCR Master Mix*	25 µl	1x
10x primer mix, 2 µM each primer (see Table 2)	5 µl	0.2 µM†
Q-Solution, 5x	5 µl	0.5x
RNase-free water	Variable	–
Template DNA		
Added at step 4	Variable	≤1 µg DNA/50 µl
Total volume	**50 µl‡**	

* Provides a final concentration of 3 mM $MgCl_2$.

† A final primer concentration of 0.2 µM is optimal for most primer–template systems. However, in some cases using other primer concentrations (0.1–0.3 µM) may further improve amplification performance.

‡ For volumes less than 50 µl, the 1:1 ratio of QIAGEN Multiplex Master Mix to primer mix, template, and Q-Solution should be maintained.

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

   Mix gently, for example by pipetting the reaction mix up and down a few times. Due to the hot start, it is not necessary to keep samples on ice during reaction setup.

4. **Add template DNA (≤1 µg/50 µl reaction) to the individual PCR tubes containing the reaction mix.**

   For multiplex RT-PCR, the volume of cDNA added (from the RT reaction) as template should not exceed 10% of the final PCR volume.

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

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

   Optional: If a thermal cycler with a temperature gradient function can be used, determine the optimal annealing temperature by performing a gradient PCR.

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Table 13. Universal Multiplex Cycling Protocol

			Additional comments
Initial activation step:	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:			
Denaturation	30 s	94°C	
Annealing	**90 s**	57–63°C	**If a gradient PCR cannot be performed, use 60°C as the starting annealing temperature. If the lowest T_m* of your primer mixture is below 60°C, use 57°C as starting annealing temperature.**
Extension	90 s	72°C	Optimal for targets up to approximately 1.5 kb in length.†
Number of cycles	30–45		The number of cycles is dependent on the amount of template DNA and the required sensitivity of your detection method. See Appendix C, page 36 for guidelines.
Final extension:	10 min	72°C	

* T_m determined according to the formula: T_m = 2°C x (number of [A+T]) + 4°C x (number of [G+C]).

† For targets longer than 1.5 kb, an extension time of 2 min may improve performance.

7. **Place the PCR tubes in the thermal cycler and start the cycling program as outlined in Table 13.**

   Each PCR program must start with an initial heat-activation step at 95°C for 15 min to activate HotStarTaq DNA Polymerase.

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

8. **Analyze samples using an appropriate detection system, for example agarose gel electrophoresis (see Table 3, page 10 for choosing the optimal percentage of agarose), polyacrylamide gel electrophoresis*, or capillary electrophoresis.**

   The optimal amount of PCR product required to give a satisfactory signal with your detection method should be determined individually.

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

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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 DNA Polymerase not activated	Ensure that the cycling program included the HotStarTaq DNA Polymerase activation step (15 min at 95°C) as described in step 7 of the protocols (pages 18, 22, and 25).
b)	Pipetting error or missing reagent	Repeat the PCR. Check the concentrations and storage conditions of reagents, including primers and template DNA. Mix all solutions before use.
c)	Primer concentration not optimal	Use a concentration of 0.2 µM of each primer. For amplification of long targets (≥1.5 kb), a primer concentration of 0.1 µM and extension time of 2 min may improve results. We do not recommend using primer concentrations higher than 0.3–0.4 µM, as this may affect multiplex PCR fidelity. Check concentration of primer stock solutions. For calculation of primer concentration, refer to Appendix B, page 34.
d)	Insufficient number of cycles	Increase number of PCR cycles. Refer to Appendix C, page 35 for guidelines.
e)	PCR cycling conditions not optimal	Check that the correct cycling conditions were used (see Tables 9, 11, and 13 on pages 19, 22, and 25, respectively). Ensure that an annealing time of 90 s was used. If possible, perform a gradient PCR to determine the optimal annealing temperature (see Appendix C, page 36).
f)	PCR cycling conditions not optimal	Check functionality and specificity of primer pairs in single reactions. Ensure that primers of sufficiently high quality were used. Check for possible degradation of the primers on a denaturing polyacrylamide gel*. If necessary, make new dilutions of primer mix from primer stock solutions and store at –20°C in small aliquots. Avoid repeated freeze–thaw cycles of the primer mix.

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

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g)	Annealing temperature too high	Follow the recommendations given in Appendix A, page 33 to determine the appropriate annealing temperature for your primers. Decrease annealing temperature in increments of 3°C. Ensure that an annealing time of 90 s was used. If possible, perform a gradient PCR (see Appendix C, page 36) to determine the optimal annealing temperature.
h)	GC-Rich template or template with a high degree of secondary structure	Using the same cycling conditions, repeat the multiplex PCR using 0.5x Q-Solution. Follow the protocol on page 22. Templates with a very high GC content that do not amplify under these conditions should be combined in a separate multiplex PCR assay using 1x Q-Solution.
i)	Primer design not optimal	Review primer design. Refer to Appendix A, page 33 for general guidelines on multiplex PCR primer design.
j)	Insufficient starting template	Increase amount of starting template up to 1 µg per 50 µl reaction.
k)	Low-quality starting template	Use only high-quality DNA, such as that purifed using DNeasy kits (see page 11).
l)	Problems with starting template	Check the concentration, storage conditions, and quality of the starting template (see Appendix B, page 34). If necessary, make new serial dilutions of template nucleic acid from stock solutions. Repeat the multiplex PCR using the new dilutions.
m)	PCR product too long	The optimized protocols allow amplification of target sequences up to 1.5 kb. We recommend using an extension time of 2 min for target sequences between 1.5–2.0 kb. Increase the extension time in increments of 30 s for each additional 0.5 kb of target sequence.
n)	PCR overlaid with mineral oil when using a thermal cycler with a heated lid	When performing PCR in a thermal cycler with a heated lid, do not overlay the PCR samples with mineral oil if the heated lid is switched on. This may decrease the yield of PCR product.
o)	Problems with the thermal cycler	Check the power to the thermal cycler and that the thermal cycler has been correctly programmed.

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Not all products are detectable, or some products are barely detectable

a)	Primers degraded or of-low quality	Check functionality and specificity of primer pairs in single reactions. Ensure that primers of sufficiently high quality were used. Check for possible degradation of the primers on a denaturing polyacrylamide gel*. If necessary, make new dilutions of primer mix from primer stock solutions and store at –20°C in small aliquots. Avoid repeated freeze–thaw cycles of the primer mix.
b)	Primer concentration not optimal	Use a primer concentration of 0.2 µM. For amplification of long targets (≥1.5 kb), a primer concentration of 0.1 µM may improve results. We do not recommend using primer concentrations higher than 0.3–0.4 µM, as this may affect multiplex PCR fidelity. Check concentration of primer stock solutions (see Appendix B, page 34).
c)	PCR cycling conditions not optimal	Check that the correct cycling conditions were used (see Tables 9, 11, and 13 on pages 19, 22, and 25, respectively). Ensure that an annealing time of 90 s was used. If possible, perform a gradient PCR to determine the optimal annealing temperature (see Appendix C, page 36).
d)	No final extension step, or final extension step was not optimal	Ensure that the final extension step was performed as described in Tables 8, 10, and 12 on pages 19, 22, and 25, respectively. When detecting multiplex PCR products under native conditions, a final extension step of 15 min at 68°C for multiplex systems with more than 10 PCR products, or for PCR products longer than 1.5 kb may improve results. For microsatellite analysis, a final extension step of 30 min at 60°C should be used.
e)	Annealing temperature too high	Check that the correct cycling conditions were used (see Tables 9, 11, and 13 on pages 19, 22, and 25, respectively). Ensure that an annealing time of 90 s was used. If possible, perform a gradient PCR to determine the optimal annealing temperature (see Appendix C, page 36).
f)	GC-rich template or template with a high degree of secondary structure	Using the same cycling conditions, repeat the multiplex PCR using Q-Solution. Follow the protocol on page 23. Templates with a very high GC content that do not amplify under these conditions should be combined in a separate multiplex PCR assay using 1x Q-Solution.

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

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g)	Sensitivity not high enough	If your assay requires very high sensitivity, the sensitivity of the multiplex PCR can be further increased by an extended annealing time of 3 min.

Additional products detectable

a)	PCR cycling conditions not optimal	Check that the correct cycling conditions were used (see Tables 9, 11, and 13 on pages 19, 22, and 25, respectively). Ensure that an annealing time of 90 s was used. If possible, perform a gradient PCR to determine the optimal annealing temperature (see Appendix C, page 36).
b)	Too many PCR cycles	Too many PCR cycles may increase nonspecific background. Determine the optimal number of cycles by decreasing the number of PCR cycles in increments of 3 cycles.
c)	Annealing temperature too low	Follow the recommendations given in Appendix A, page 33 to determine the appropriate annealing temperature for your primers. Increase annealing temperature in increments of 2°C. Ensure that an annealing time of 90 s was used. If possible, perform a gradient PCR to determine the optimal annealing temperature (see Apppendix C, page 36).
d)	Mg^{2+} concentration not optimal	Use an initial Mg^{2+} concentration of 3 mM as provide by the QIAGEN Multiplex PCR Master Mix. In rare cases, an increase in Mg^{2+} concentration may increase product yield. Perform multiplex PCR with different final concentrations of Mg^{2+} by titrating in 0.5 mM steps.
e)	Primer concentration not optimal	Use a primer concentration of 0.2 µM. For amplification of long targets (≥1.5 kb), a primer concentration of 0.1 µM may improve results. We do not recommend using primer concentrations higher than 0.3–0.4 µM, as this may affect multiplex PCR fidelity. Check concentration of primer stock solutions. For calculation of primer concentration, refer to Appendix B, page 34.
f)	Primer design not optimal	Review primer design. Refer to Appendix A, page 33, for general guidelines on multiplex PCR primer design.
g)	Some Primers generate more than one specific product	Multiplex primer pairs bind in close proximity to each other, for example during amplification of multiple parts of a genomic locus. Additional larger products may be generated by outside primers (see Appendix E, page 37).

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h)	Primers degraded or of low quality	Check functionality and specificity of primer pairs in single reactions. Ensure that primers of sufficiently high quality were used. Check for possible degradation of the primers on a denaturing polyacrylamide gel*. If necessary, make new dilutions of primer mix from primer stock solutions and store at –20°C in small aliquots. Avoid repeated freeze–thaw cycles of the primer mix.
i)	Amplification of pseudogene squences	rimers may anneal to pseudogene sequences and additional PCR products may be amplified. Review primer design to avoid detection of pseudogenes. Refer to Appendix A, page 33 for general guidelines on multiplex PCR primer design.
j)	Not final extension step, or final extension step not optimal	Ensure that the final extension step was performed as described in Tables 9, 11, and 13 on pages 19, 22, and 25, respectively. When detecting multiplex PCR products under native conditions, a final extension step of 15 min at 68°C for multiplex systems with more than 10 PCR products, or for PCR products longer than 1.5 kb may improve results. For microsatellite analysis, a final extension step of 30 min at 60°C should be used.
k)	GC-rich template or template with a high degree of secondary structure	Using the same cycling conditions, repeat the multiplex PCR using Q-Solution. Follow the protocol on page 23. Templates with a very high GC content that do not amplify under these conditions should be combined in a separate multiplex PCR assay using 1x Q-Solution.

If detecting multiplex PCR products under non-denaturing conditions (e.g., on agarose gels or native polyacrylamide gels)*

Some products are smeared, or additional products are observed

a)	Too many PCR cycles	Too many PCR cycles may increase nonspecific background. Determine the optimal number of cycles by decreasing the number of PCR cycles in increments of 3 cycles.
b)	Too much starting template	Check the concentration of the starting template DNA (see Table 6, page 13). Repeat the multiplex PCR using less DNA (i.e., ≥1 µg per 50 µl reaction).

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

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c)	No final extension step, or final extension step was not optimal	Ensure that the final extension step was performed as described in Tables 9, 11, and 13 on pages 19, 22, and 25, respectively. When detecting multiplex PCR products under native conditions, a final extension step of 15 min at 68°C for multiplex systems with more than 10 PCR products, or for PCR products longer than 1.5 kb may improve results. For microsatellite analysis, a final extension step of 30 min at 60°C should be used.
d)	Incomplete renaturation of PCR products due to either low GC content or long length of PCR products	Use a final extension step of 15 min at 68°C. We recommend this for multiplex systems with more than 10 PCR products, or for PCR products longer than 1.5 kb.
e)	Double-stranded products melt during electrophoresis	PCR products with a low GC content may melt if electrophoresed at high voltages. Reduce the voltage to prevent the running buffer from overheating.
f)	Background using silver staining	Purify PCR products before loading onto the gel (e.g., using the QIAquick® Gel Extraction Kit or MinElute® Gel Extraction Kit).

If detecting fluorescently labeled multiplex PCR products under denaturing conditions (e.g., on an automatic sequencer, or by capillary electrophoresis)

Additional products are observed

a)	Loading volume is too high	Loading of large volumes may result in additional peaks. Decrease the loading volume until the background is decreased to a satisfactory level with acceptable peak heights (typically peak heights <2000 relative fluorescent units on ABI PRISM® 310 or 377 Genetic Analyzer).
b)	Faint peaks (“stutter peaks”) before main peak	Amplification of some microsatellite DNA sequences may lead to artifacts, referred to as stutter peaks, which are usually one repeat unit shorter than the main peak. We recommend decreasing the loading volume as described above. If the length of the faint peak is one base shorter than the main peak, refer to “n–1 products detected” below.
c)	Sample not completely denatured	Denature the samples before loading by heating to 95°C for 3 min.

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d)	n–1 products detected	Ensure that the final extension step was performed as described in Tables 8, 10, and 12 on pages 19, 22, and 25, respectively. If the final extension step was correctly performed, decrease the number of cycles and/or template amount.
e)	Differing signal intensities from fluorescent dyes	Different fluorescent dyes may give differing signal intensities on a particular detection instrument, although comparable amounts of PCR product are generated. We recommend combining fluorescent dyes for multiplex PCR according to the instructions of the detection instrument's manufacturer.
Faint peaks or no allele peaks		
a)	Poor capillary electrophoresis injection (size standard also affected)	Inject the sample again. Check the syringe O-ring for leakage. Check that the fluorescence detection instrument is functioning correctly.
b)	Poor quality formamide used	Use high-quality formamide for the analysis of samples on the ABI PRISM 310 Genetic Analyzer. The conductivity of the formamide should be <100 µS/cm.
If performing RT-PCR		
Little or no product		
RT reaction error		On average only 10–30% of the starting RNA in the RT reaction is transcribed into cDNA. The volume of the RT reaction added as template should not exceed 10% of the final PCR volume.
Additional larger products		
Contamination with genomic DNA		Additional larger products may result from amplification of contaminating genomic DNA. Pretreat RNA with DNase I (e.g., using the QIAGEN RNase-Free DNase Set, cat no. 79254). Alternatively, use primers located at splice junctions of the target mRNA to avoid amplification from genomic DNA.

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Appendix A: Design of Multiplex PCR Primers

A prerequisite for successful multiplex PCR is the design of optimal primer pairs.

- Primers for multiplex PCR should be 21–30 nucleotides in length.
- Primers for multiplex PCR should have a GC content of 40–60%.

The probability that a primer has more than one specific binding site within a genome is significantly lower for longer primers. In addition, longer primers allow annealing at slightly higher temperatures where *Taq* DNA polymerase activity is higher.

Melting temperature (T_m)

- The melting temperature of primers used for multiplex PCR should be at least 60°C. For optimal results, we recommend using primer pairs with a T_m of ≥68°C. Above 68°C, differences in T_m values of different primer pairs do not usually affect performance.

The melting temperature of primers can be calculated using the formula below:

T_m = 2°C x (number of [A+T]) + 4°C x (number of [G+C])

- Whenever possible, design primer pairs with similar T_m values. Functionality and specificity of all primer pairs should be checked in individual reactions before com bining them in a multiplex PCR assay.

Annealing temperature

If possible, perform a gradient PCR to determine the optimal annealing temperature (see page 36). Otherwise, use the recommendations in Table 13.

Table 13. Recommended Annealing Temperatures for Multiplex PCR

Lowest primer T_m	Annealing temperature
<60°C	Perform gradient PCR over the range 48–60°C
60–66°C	57–60°C
68°C	60–63°C

Sequence

When designing primers for multiplex PCR the following points should be noted:

- Avoid complementarity of 2 or 3 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 and/or C at the 3' end.

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- Avoid complementary sequences within primers and between primer pairs.
- Ensure primer sequence is unique for your template sequence. Check similarity to other known sequences with BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Commercially available computer software (e.g., OLIGO 6, Rychlik, 1999) or web-based tools such as Primer3, Steve Rosen & Helen Skaletsky, 2000, (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) can be used for primer design.

Distinguishing individual PCR products

Depending on your method of detection, primers should be chosen so that the corresponding PCR products can be easily distinguished from one another, for example by size difference (see Tables 3 and 5), or by using primers labeled with different fluorescent dyes.

Appendix B: Handling and Storage of Primers

Determining primer concentration and quality

Primer quality is crucial for successful multiplex PCR. Problems encountered with multiplex PCR are frequently due to incorrect concentrations of primers being used. If you observe large differences in yield of different amplification products in a multiplex PCR, check that all primers were used at the correct concentration. For optimal results, we recommend only combining purified primers of comparable quality.

Dissolving primers

- Lyophilized primers should be dissolved in a small volume of low salt buffer to make a concentrated stock solution. We recommend using TE* (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) for standard primers and primers labeled with most fluorescent dyes.
- Since they tend to degrade at higher pH, primers labeled with fluorescent dyes such as Cy®3, Cy3.5, Cy5, and Cy5.5, should be stored in TE, pH 7.0.
- Before opening tubes containing lyophilized primers, spin tubes briefly to collect all material at the bottom of the tube.
- To dissolve the primer, add the volume of TE quoted on the oligo vial or datasheet, mix, and leave for 20 minutes to let the primer completely dissolve. We do not recommend dissolving primers in water. Primers are less stable in water than TE and some primers may not dissolve easily in water.

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

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Quantification of primers

The given amount and/or concentration after dissolving of commercially supplied primers is often a very rough approximation. Before use, primers should be accurately quantified using a spectrophotometer. After dissolving the primer using the volume of TE quoted on the oligo vial or datasheet, measure the A_{260} (OD) of a 1 in 100 dilution of the stock solution using a glass cuvette with a 1 cm path-length, and calculate the concentration.* This measured value should be used for subsequent calculations.

Spectrophotometric conversion for primers: 1 A_{260} unit (1 OD) = 20–30 µg/ml

Concentration can be derived from the molar extinction coefficient (ε_{260}) and A_{260}(OD)

$$A_{260}\ (\text{OD}) = \varepsilon_{260} \times \text{molar concentration of the primer}$$

If the ε_{260} value is not given on the primer data sheet, it can be calculated from the primer sequence using the following formula:

$$\varepsilon_{260} = 0.89 \times [(n\text{A} \times 15{,}480) + (n\text{C} \times 7340) + (n\text{G} \times 11{,}760) + (n\text{T} \times 8850)]$$

where n = number of respective bases.

Example

Primer length: 24 nucleotides with 6 each of A, C, G, and T bases.

Observed A_{260} (OD) of a 1 in 100 dilution = 0.283

$\varepsilon_{260} = 0.89 \times [(6 \times 15{,}480) + (6 \times 7340) + (6 \times 11{,}760) + (6 \times 8850)] = 231{,}916$

Concentration = A_{260} (OD)/ε_{260} = 0.283/231,916 = 1.22×10^{-6} M = 1.22 µM

Concentration of primer stock solution = concentration of dilution x dilution factor

= 1.22 µM x 100 = 122 µM

Creating normalized primer stock solutions for the 10x primer mix

Depending on the level of multiplexing in the reaction, determine whether the required concentration of the normalized primer stock solution is 50 µM or 100 µM (Table 2, page 9).

Calculate the required dilution factor = required concentration/actual concentration

To produce 100 µl of the desired primer concentration, pipet X µl (where X = dilution factor x 100) of the stock solution into a clean tube and make up to 100 µl with TE.

Example

To create 100 µl of a 50 µM normalized primer stock solution using the primer from the example above:

Dilution factor = 50 µM/122 µM = 0.41

Pipet 0.41 x 100 = 41 µl stock solution into a tube and add 59 µl TE to give a 50 µM normalized primer stock solution.

* To ensure significance, A_{260} readings should be greater than 0.15.

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Primer quality

The quality of 18–30mers can be checked on a 15% denaturing polyacrylamide gel*; a single band should be seen. Please call one of the QIAGEN Technical Service Departments or local distributors for a protocol (see back cover) or visit www.qiagen.com .

Storage

Primers should be stored in TE in small aliquots at –20°C. Unmodified primers are stable under these conditions for at least one year and fluorescently labeled primers are usually stable under these conditions for at least 6 months. Repeated freeze–thaw cycles should be avoided since they may lead to primer degradation.

For easy and reproducible handling of the numerous primers used in multiplex PCR, we recommend the preparation of a 10x primer mix containing all primers necessary for a particular multiplex PCR assay at equimolar concentrations (see Table 2, page 9).

Appendix C: PCR Cycling Protocols

A cycling program usually consists of 25–45 cycles, depending on the number of copies of the starting template and the sensitivity of the detection system used. Increasing the number of cycles does not necessarily lead to a higher yield of multiplex PCR product; instead it may increase nonspecific background and may lead to artifacts. Table 15 provides general guidelines for choosing the number of cycles. We recommend starting with the lowest number of cycles given for a particular template amount.

Table 15. General Guidelines for Choosing the Number of PCR Cycles

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

* Refers to single-copy genes

Gradient PCR

Many thermal cyclers have a temperature-gradient function. Using this function, it is possible to easily determine optimal annealing temperatures by generating a temperature gradient across the heating block for the annealing step.

If your primers conform to the criteria on page 33, we recommended using a gradient program that includes a temperature range from 50–70°C. In order to determine optimal annealing conditions, prepare 3 identical reactions and place in the block positions that most closely correspond to annealing temperatures of 57, 60, and 63°C.

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

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Analysis using fluorescence detection systems

Fewer PCR cycles are needed to generate sufficient product for analysis using fluorescence detection instruments, such as automatic sequencers based on gel or capillary electrophoresis. The optimal number of PCR cycles and the amount of PCR product that is used for analysis is dependent on the sensitivity of the detection system and should be determined individually. We recommend using 10–12 fewer cycles than the lowest number given in Table 15 for a particular template amount. Too many PCR cycles may lead to artifacts or additional products and should be avoided.

Appendix D: Analysis of Multiplex PCR Products

The concentration of the agarose gel for separation of multiplex PCR products should be appropriate for the overall size of products generated and should be suitable for resolving the small size differences between PCR products (see Table 3, page 10). For optimal results, we recommend the use of 1x TAE* buffer for preparation and running of the gel.

Fluorescently labeled primers

Fluorescent labels should be chosen so that they are compatible with your detection instrument. We recommend choosing fluorescent labels according to the manufacturer of your detection instrument's instructions. See Appendix B, page 34 for general guidelines on handling and storage of fluorescently labeled primers.

Appendix E: Special Multiplex PCR Applications

Sensitive multiplex PCR assays

PCR is an exquisitely sensitive technique that can be used to amplify and detect even 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 or by the amount and quality of template DNA. The combination of HotStarTaq DNA Polymerase and QIAGEN Multiplex PCR Buffer increases specificity both at the start of and during PCR, making the QIAGEN Multiplex PCR Kit well-suited for highly sensitive PCR assays. Sensitivity can be further increased when using very low amounts of DNA (20 copies), by increasing the annealing time from 90 seconds to 3 minutes. However, some alleles may not be detectable when using low amounts of template DNA, due to the fact that not all target loci are present in the reaction. This is caused by stochastic fluctuation and we therefore recommend using a minimum of 20 copies of template DNA per reaction.

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

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Transgene detection

Genetically modified animals and plants can be generated to study the function of particular genes. Targeted mutations can be introduced that alter the function of the gene locus of interest, for example by inactivating or modifying its function. This allows research on the role of certain genes in complex biological processes. Modified genomic loci can be easily distinguished by multiplex PCR. To distinguish the wild-type from the mutant gene locus, pairs of primers should be designed that are specific either for the wild-type locus or for the mutant locus. In Figure 2, one primer is located on the wild-type locus. Two reverse primers have been designed, so that one primer is specific for the wild-type locus and the other specific for the mutant locus, resulting in PCR products of different sizes. Multiplex PCR is highly suited for the efficient detection of transgenes. In this case, two primer pairs can be used: one pair specific for the introduced transgene and the other pair specific for a wild-type DNA sequence. This second primer pair acts as a control for the amount and quality of the template DNA.

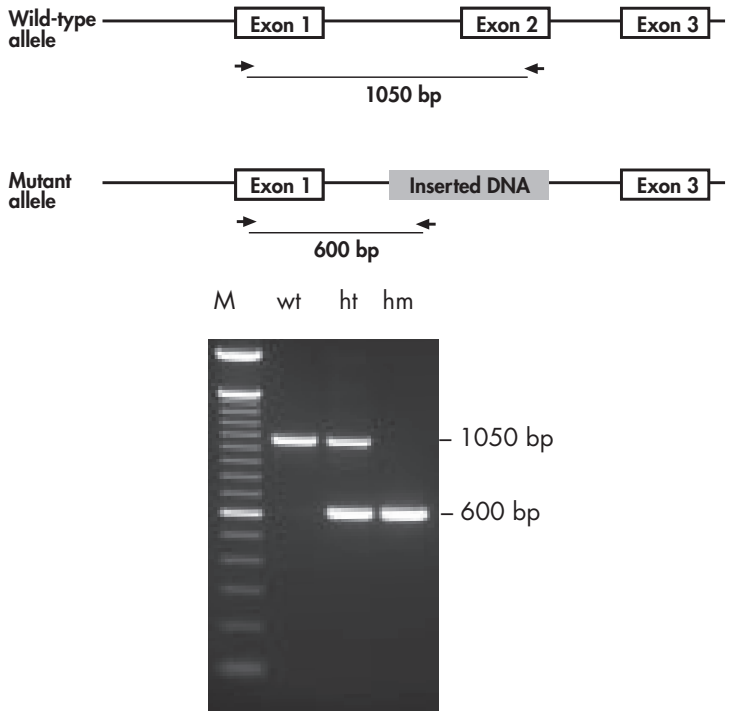

Figure 2. Detection of Transgenes. Transgenic mice were screened using the QIAGEN Multiplex PCR Kit and a set of 3 primers specific for the recombination activating gene 2 locus. The primers were designed to generate PCR products of different sizes so that wild-type mice (**wt**) could be easily distinguished from heterozygote mutant mice (**ht**), or homozygote mutant mice (**hm**). **M**: markers.

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Analysis of microsatellites

Eukaryotic genomes contain repeated DNA sequences that differ both in the length and sequence of the repeat. These repeats, known as satellite DNA, are often highly polymorphic, with many different alleles present within a population. Alleles may also differ in the number of copies of a particular repeat sequence. Variation in satellite DNA sequences is important and can be used to determine genetic differences between organisms or closely related individuals. Satellite loci used for PCR analysis are usually short repeat units known as microsatellites. The length of the repeat unit is typically 2–6 nucleotides, and is referred to as a short tandem repeat (STR). In some cases, slightly longer repeats are used for analysis. These are referred to as variable number of tandem repeats (VNTRs) and can vary in both repeat length and number of repeats.

Amplification of microsatellite DNA may result in a low percentage of PCR products that are shorter than expected. Typically, the difference in size of these "stutter products" is exactly one repeat unit and these products are an intrinsic feature of the amplification of some satellite DNA sequences.

Taq DNA polymerases, including HotStarTaq in the QIAGEN Multiplex PCR Master Mix, add an extra A residue to the 3' end of PCR products. When using the microsatellite cycling protocol given on page 20, all PCR products should have this additional A residue at the 3' end. However, if larger amounts of PCR product are generated using an increased number of cycles, it may be possible that not all PCR products will have the extra A residue and some n–1 product may be detectable. This may interfere with data analysis, depending on the resolution of the detection system. Decreasing the number of cycles can help to minimize the number of n–1 products.

Multiplex PCR of microsatellite loci is frequently performed using fluorescently labeled primers. Ensure that the fluorescent labels are compatible with the detection system used. Different fluorescent dyes may give differing signal intensities on a particular detection instrument, although comparable amounts of PCR product are generated. We recommend combining fluorescent dyes for multiplex PCR according to the detection instrument's instructions. Analysis of microsatellite DNA is usually performed using nanogram amounts of template DNA. However, in some cases only smaller amounts of DNA may be available. The QIAGEN Multiplex PCR Kit is highly suited for performing microsatellite analysis with low amounts (≤0.1 ng) of genomic DNA. Increasing the annealing time from 90 seconds to 3 minutes can further increase the sensitivity of the reaction, and gives very reliable genotyping results. With such low amounts of template, some alleles may not be detectable due to the fact that not all target loci are present in the reaction. This is caused by stochastic fluctuation.

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SNP analysis

Single nucleotide polymorphisms (SNPs) are single nucleotide changes occurring at specific points within the genome. Analysis of SNPs is important, because they are a genetic marker that can be used to associate genetic changes with disease. Study of SNPs can also be used to analyze complex phenotypes or to establish whether a genetic relationship exists between certain individuals. To provide sufficient material for analysis, the regions carrying the relevant SNPs can be amplified from genomic DNA by multiplex PCR, using the QIAGEN Multiplex PCR Kit. After amplification, primers, unincorporated nucleotides, enzyme, salts, and mineral oil may need to be removed before the specific PCR products can be used in subsequent experiments. The QIAquick system offers a quick and easy method for purifying PCR products. 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, call your local QIAGEN Technical Service Department or distributor (see back cover for contact information or visit www.qiagen.com).

Detection of genetically modified organisms or microorganisms

Multiple organisms can be detected simultaneously using multiplex PCR. In comparison to established methods, the QIAGEN Multiplex PCR Kit provides higher sensitivity of amplification due to the optimized QIAGEN Multiplex PCR buffer in combination with HotStarTaq polymerase. This allows amplification of multiple products with differing copy numbers in the multiplex PCR . See page 37 for more information on amplification of sequences from low amounts of DNA and also for information on sensitive multiplex PCR assays.

Gene expression analysis

Although an organism's cells generally contain the same genetic information, However, as a result of cell differentiation, many genes are differentially expressed. Multiplex PCR using cDNA as a template can be used for qualitative and semi-quantitative analysis of differentially expressed genes. RNA must be reverse transcribed using an appropriate reverse transcriptase (e.g., Omniscript or Sensiscript RT). Since components of the reverse transcriptase reaction can interfere with the subsequent PCR, the volume of the reverse transcriptase reaction added to the PCR should not exceed 10% of the final volume.

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Exon-specific PCR

Multiplex PCR is frequently used for amplification of multiple regions of a gene, for example to detect regions carrying SNPs or other mutations. Since the outside primers in the multiplex PCR may still be quite close to each other, additional larger PCR products may be amplified from these outside primers (Figure 3). The generation of the additional, larger PCR products can be prevented by a digestion of the template DNA with a suitable restriction enzyme that cuts the template DNA between the affected fragments but not within the amplified regions.

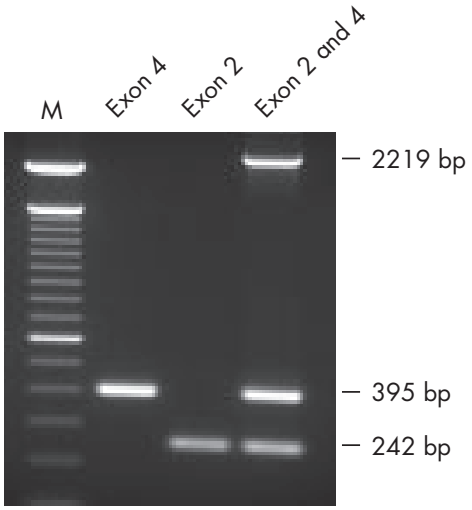

Figure 3. Generation of Additional PCR Products in Exon-Specific PCR. Two exons from the human HFE gene were amplified from human genomic DNA either separately, (**Exon 4**); (**Exon 2**), or together (**Exon 2 and 4**). The PCR products generated from Exon 4 and Exon 2 were 395 bp and 242 bp respectively. However, in the multiplex PCR an additional product of 2219 bp is observed, generated by the 2 outside primers. **M**: markers.

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Appendix F: Optimization of Reaction Conditions for Special Multiplex PCR Applications

The QIAGEN Multiplex PCR Kit protocols have been developed to give satisfactory results in most cases. In some special cases, modifications to the conditions given in the protocol may improve performance.

Large number of PCR products

For multiplex amplification reactions with more than 10 PCR products, a decrease of primer concentration to 0.1 µM may lead to a more uniform product yield. The annealing time can also be increased from 90 seconds to 3 minutes. When detecting multiplex PCR products under native conditions, a final extension step of 15 minutes at 68°C may improve results. To establish a multiplex system with a large number of PCR products, it is strongly recommended to check the primer concentration given by the primer supplier (see page 34). The use of high-quality (e.g., HPLC purified) primers is recommended, although standard quality may also be sufficient. We strongly recommend only combining primers of comparable quality.

Highly sensitive applications or low template amounts

Increasing annealing time from 90 seconds to 3 minutes may further increase sensitivity.

Uniform product yield and signal intensity

If the PCR products are not generated uniformly, check the concentration of all primers used in the multiplex assay (see page 34). Differences in primer concentration due to incorrect quantification or dilution are the most likely cause of nonuniform product yield. To further improve signal intensity, the concentration of the primers can be reduced to 0.1 µM by using half of the original volume of 10x primer mix (see Table 2, page 9) and the concentration of the primers yielding low amounts of product can be increased to 0.3 µM.

A primer participates in more than one reaction

If a primer participates in more than one reaction, (e.g., as described for transgene detection, page 38), doubling the concentration of this primer to 0.4 µM may lead to more uniform product yield.

Long PCR products (≥1.5 kb)

For PCR products greater than 1.5 kb in length, optimal results may be obtained by decreasing the primer concentration to 0.1 µM. The annealing time can also be increased from 90 seconds to 3 minutes. For detection of PCR products under native conditions (e.g., by agarose gel electrophoresis), a final extension step of 15 minutes at 68°C may improve results.

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Shortening the cycling program

If the length of the cycling program is critical and you wish to shorten it, we recommend decreasing the extension time in increments of 15 seconds. We strongly recommend maintaining an annealing time of 90 seconds. The PCR program should start with an initial heat-activation step of 15 minutes at 95°C.

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Ordering Information

Product	Contents	Cat. no.
QIAGEN Multiplex PCR Kits — for fast and efficient multiplex PCR		
QIAGEN Multiplex PCR Kit (100)	For 100 x 50 µl multiplex reactions: 2x QIAGEN Multiplex PCR Master Mix (containing 6 mM $MgCl_2$, 3 x 0.85 ml), 5x Q-Solution (1 x 2.0 ml), RNase-free water (2 x 1.7 ml)	206143
QIAGEN Multiplex PCR Kit (1000)	For 1000 x 50 µl multiplex reactions: 2x QIAGEN Multiplex PCR Master Mix (containing 6 mM $MgCl_2$, 1 x 25 ml), 5x Q-Solution (1 x 10 ml), RNase-free water (2 x 20 ml)	206145
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 QSolution, 25 mM $MgCl_2$	200203
HotStarTaq *Plus* DNA Polymerase — for highly specific hot-start PCR		
HotStarTaq *Plus* DNA Polymerase (250)*	250 units HotStarTaq *Plus* DNA Polymerase, 10x PCR Buffer, 10x CoralLoad PCR Buffer, 5x Q-Solution, 25 mM $MgCl_2$	203603
DNeasy Tissue Kits — for isolation of up to 40 µg genomic DNA from animal tissues and cells, yeast, or bacteria		
DNeasy Tissue Kit (50)*	For 50 DNA preps: 50 DNeasy Spin Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	69504
DNeasy Plant Mini Kits — for isolation of total cellular DNA from plant cells and tissues, or fungi		
DNeasy Plant Mini Kit (50)*	For 50 DNA preps: 50 DNeasy Mini Spin Columns, 50 QIAshredder™ Mini Spin Columns, RNase A, Buffers, Collection Tubes (2 ml)	69104

[††] Contains 15 mM $MgCl_2$.

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

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Ordering Information

Product	Contents	Cat. no.
QIAamp DNA Mini Kits — for isolation of genomic, mitochondrial, bacterial, parasite, or viral dNA		
QIAamp DNA Mini Kit (50)*	For 50 DNA preps: 50 QIAamp Mini Spin Columns, Proteinase K, Reagents, Buffers, Collection Tubes (2 ml)	51304
RNeasy Kits — for purification of total RNA from animal cells or tissues, yeast, or bacteria		
RNeasy Mini Kit (50)*	For 50 RNA preps: 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
Omniscript Kits — for standard reverse transcription with any amount of RNA from 50 ng to 2 µg per reaction		
Omniscript RT Kit (50)*	For 50 reverse-transcription reactions: 200 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP mix,† RNase-free water	205111
Sensiscript Kits — for standard reverse transcription with small amounts of RNA (i.e., less than 50 ng RNA including carrier RNA)		
Sensiscript RT Kit (50)*	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP mix,† RNase-free water	205211
QIAquick Gel Extraction Kits — for gel extraction or cleanup of DNA (70 bp to 10 kb) from enzymatic reactions		
QIAquick Gel Extraction Kit (50)*	For 50 cleanup procedures: 50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28704
MinElute Gel Extraction Kits — for gel extraction of DNA fragments (70 bp to 4 kb) in low elution volumes		
MinElute Gel Extraction Kit (50)*	For 50 cleanup procedures: 50 MinElute Spin Columns, Buffers, Collection Tubes (2 ml)	28604

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Ordering Information

Product	Contents	Cat. no.
QIAxcel System — for effortless automated DNA fragment and RNA analysis		
QIAxcel System	Capillary electrophoresis device, including computer, and BioCalculator Analysis software; 1-year warranty on parts and labor	9001421
QIAxcel kits—for fast high-resolution capillary electrophoresis		
QIAxcel DNA High Resolution Kit (1200)	QIAxcel DNA High Resolution Gel Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips	929002
QIAxcel DNA Screening Kit (2400)	QIAxcel DNA Screening Gel Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips	929004
QIAxcel DNA Large Fragment Kit (600)	QIAxcel DNA Large Fragment Gel Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips	929006

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