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QIAGEN® Multiplex PCR *Plus* Handbook

For fast, efficient, and optimization-free multiplex PCR for advanced applications

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Contents

Kit Contents

QIAGEN Multiplex PCR *Plus* Kit	**(30)**	**(100)**	**(1000)**
Catalog no.	**206151**	**206152**	**206155**
Number of 50 µl reactions	**30**	**100**	**1000**
Multiplex PCR Master Mix, 2x*	1 x 0.85 ml	3 x 0.85 ml	1 x 25 ml
Q-Solution®, 5x	1 x 2 ml	1 x 2 ml	1 x 10 ml
RNase-Free Water	1 x 1.9 ml	2 x 1.9 ml	1 x 20 ml
CoralLoad® Dye, 10x	1 x 1.2 ml	1 x 1.2 ml	1 x 5.5 ml

* Contains optimized concentrations of HotStarTaq® *Plus* DNA Polymerase, $MgCl_2$, and dNTPs and Multiplex PCR *Plus* Buffer (with Factor MP).

Storage

The QIAGEN Multiplex PCR *Plus* Kit is shipped on dry ice. It should be stored immediately upon receipt at –30 to –15°C in a constant-temperature freezer. When stored under these conditions and handled correctly, the product can be kept at least until the expiration date (see the inside of the kit lid) without showing any reduction in performance. The 2x Multiplex PCR Master Mix can be stored at 2–8°C for up to 2 months without showing any reduction in performance.

Intended Use

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

Safety Information

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

Product Specifications

2x Multiplex PCR Master Mix contains:

HotStarTaq *Plus* DNA Polymerase	HotStarTaq *Plus* DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*, cloned into *E. coli*. (Deoxynucleoside-triphosphate: DNA deoxynucleotidyl-transferase, EC 2.7.7.7). The enzyme is activated by a 5-minute, 95°C incubation step.

Buffers and reagents:

Multiplex PCR *Plus* Buffer:	Contains 6 mM $MgCl_2$; pH 8.7 (20°C).
dNTP Mix:	Contains dATP, dCTP, dGTP, dTTP; ultrapure quality
Q-Solution:	5x concentrated
CoralLoad Dye:	10x concentrated
RNase-free water:	Ultra-pure quality, PCR-grade

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the QIAGEN Multiplex PCR *Plus* Kit is tested against predetermined specifications to ensure consistent product quality.

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 semiquantitative gene expression analysis, as well as identification of mRNA splicing isoforms 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 or cDNA	Application
Plants, animals/human	Analysis of satellite DNA (e.g., STR or VNTR analysis)
	Typing of transgenic plants/animals
	Lineage analysis (e.g., of farm animals)
	GMO analysis
	Detection of pathogens
	Food analysis
	Sex determination
	Detection of mutations
	Amplification of SNP loci
	Qualitative and semi-quantitative gene analysis
	Splicing isoform identification
Bacteria/viruses	Hygiene analysis
	Detection of pathogens
	Microbial genotyping
Environmental samples	Study of metagenomes
Other	Pooling of singleplex assays (time and cost savings)
	Target enrichment for high-throughput sequencing of the ancient DNA (aDNA)

Principle and procedure

The QIAGEN Multiplex PCR *Plus* Kit is specifically developed for fast and efficient multiplex PCR for various advanced applications (see Table 1). It eliminates the need for optimization, making the development of multiplex PCR assays both simple and fast.

Fast and simple method for reliable multiplex PCR

The QIAGEN Multiplex PCR *Plus* Kit is provided in a ready-to-use master mix format based on proprietary QIAGEN Multiplex Technology. The 2x master mix provided contains preoptimized concentrations of HotStarTaq *Plus* DNA Polymerase and $MgCl_2$, dNTPs, as well as a PCR buffer specially developed for multiplex PCR reactions. The new PCR buffer contains a balanced combination of salts and additives such as Factor MP which enable comparable efficiencies for annealing and extension of all primers in the reaction (see Figure 1).

Use of a master mix format reduces time and handling steps for reaction setup and increases reproducibility by eliminating many possible sources of pipetting errors.

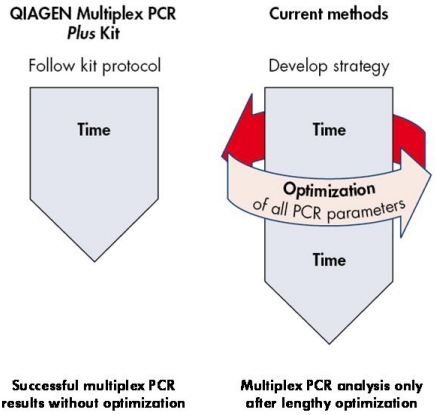

Figure 1. Successful multiplex PCR without the need for optimization. The QIAGEN Multiplex PCR *Plus* Kit is based on patented QIAGEN Multiplex Technology and provides a single, straightforward procedure for rapid and reliable results every time. In contrast to current methods, this kit eliminates the need for optimization of PCR parameters, saving time and costs.

HotStarTaq *Plus* DNA Polymerase

The Multiplex PCR Master Mix contains HotStarTaq *Plus* DNA Polymerase, a modified form of QIAGEN *Taq* DNA Polymerase. HotStarTaq *Plus* DNA Polymerase is provided in an inactive state with no polymerase activity at

ambient temperatures. This prevents the formation of misprimed products and primer–dimers during reaction setup and the first denaturation step, leading to exceptionally high PCR specificity. HotStarTaq *Plus* DNA Polymerase is activated by a 5-minute, 95°C incubation step, which is easily incorporated into existing thermal cycling programs. The hot-start enables reactions to be set up at room temperature, which is rapid and convenient.

Multiplex PCR *Plus* Buffer

The unique Multiplex PCR *Plus* Buffer facilitates the amplification of multiple PCR products. Lengthy optimization procedures, such as adjusting the amounts of enzyme, Mg^{2+}, additional reagents, and primers are virtually eliminated. In contrast to conventional PCR reagents, the Multiplex PCR *Plus* 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 of all primers in the same reaction, 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 *Plus* 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, which has been specially optimized for the requirements of multiplex PCR. It is nontoxic, and PCR purity is guaranteed.

CoralLoad Dye, 10x

The QIAGEN Multiplex PCR *Plus* Kit is supplied with CoralLoad Dye, which contains a gel loading reagent and two gel tracking dyes that facilitate estimation of DNA migration distance and optimization of agarose gel run time (Figure 2). When using CoralLoad Dye, in the multiplex PCR reaction, the amplicons can be directly loaded onto an agarose gel or the QIAxcel® Advanced System, without prior addition of loading buffer. CoralLoad dyes do not interfere with most downstream enzymatic applications. However, for reproducible results, purification of PCR products prior to enzymatic manipulation is recommended.

Note: If using capillary sequencers for detection, CoralLoad Dye must not be used.

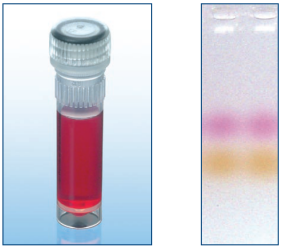

Figure 2. CoralLoad Dye for easy PCR setup and convenient DNA visualization.

Equipment and Reagents to Be Supplied by User

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

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

Important Notes

Primers

The QIAGEN Multiplex PCR *Plus* 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 reverse phase purification, HPLC purification, or related purification technologies and dissolved in TE (10 mM Tris, 1 mM EDTA, pH 8.0) to obtain a 50 or 100 µM stock solution (see Table 2, page 12). 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.

Multiplex PCR of mutation targets is sometimes performed using fluorescently labeled primers followed by subsequent detection on high-resolution sequencing instruments, such as the ABI Prism® 3100 Genetic Analyzer, Applied Biosystems® 3130 or 3130xl Genetic Analyzer, or Applied Biosystems 3730 or 3730xl DNA Analyzer. Ensure that your fluorescent labels are compatible with the detection system used. We recommend combining fluorescent dyes for multiplex PCR according to the instructions of the detection instrument's manufacturer. Different fluorescent dyes may give differing signal intensities on a particular detection instrument, although comparable amounts of PCR product are generated. Primers labeled with fluorescent dyes should always be protected from light to prevent the fluorescent dye from bleaching. The use of HPLC grade primers is recommended. See Appendix B and Appendix C, pages 38 and 43, respectively, for general guidelines on handling and storage of fluorescently labeled primers.

- The functionality of all primer pairs should be tested in singleplex reactions before combining them in a multiplex PCR mutation assay.
- For easy handling of the numerous primers used in multiplex PCR, we recommend the preparation of a primer mix containing all primers at equimolar concentrations.
- The primer mix should be prepared in TE, as described in Table 2 (page 12) 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 2 µM each primer)*

Concentration of 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 stocks) or containing up to 25 primer pairs (100 µM stocks).

† Values are valid for fluorescent and nonfluorescent primers.

Methods of analysis

Detection of targets amplified by the QIAGEN Multiplex PCR *Plus* Kit can be easily performed on various detection platforms.

Most frequently, detection on agarose gels is used. Alternatively, following amplification, amplicons can be analyzed on capillary electrophoresis instruments such as the QIAxcel Advanced System or the Agilent® 2100 Bioanalyzer, allowing resolutions of up to 3–5 bp.

Analysis of mutations on high-resolution sequencing instruments requires fluorescently labeled primers and allows resolution down to single base.

Primer pairs for multiplex PCR analysis 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 depending on the resolution of the detection system.

When using different fluorescent dyes, the PCR product can also be distinguished by the different dye label, allowing analysis of fragments of the same size in the same reaction.

Recommendations for the use of the QIAGEN Multiplex PCR *Plus* Kit with different detection systems are given in Tables 3, 4, and 5 (pages 13 and 14).

Table 3. Guidelines for agarose gel analysis of multiplex PCR products

Minimum difference in size of multiplex 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%

* The cycling protocols are designed for amplicons up to 1500 bp in length. For longer targets, please refer to the relevant recommendations in the protocol.

† 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 the QIAxcel Advanced System

QIAxcel cartridge	Application	Fragment size range	Cartridge 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 DNA High Resolution Cartridge is the recommended cartridge for analysis of multiplex PCR products obtained with the QIAGEN Multiplex PCR *Plus* Kit.

† The cycling protocols are designed for amplicons up to 1500 bp in length. For longer targets, please refer to the relevant recommendations in the protocol.

Table 5. Guidelines for analysis of multiplex PCR products using the Agilent 2100 Bioanalyzer‡

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%

‡ The cycling protocols are designed for amplicons up to 1500 bp in length. For longer targets, please refer to the relevant recommendations in the protocol.

Guidelines for analysis of multiplex PCR products on capillary sequencers

For successful analysis of multiplex PCR products derived with the QIAGEN Multiplex PCR *Plus* Kit on capillary or gel-based sequencing instruments, different instruments can be chosen:

- ABI Prism 310 or 3100 Genetic Analyzer
- Applied Biosystems 3130 or 3130xl Genetic Analyzer
- Applied Biosystems 3730 or 3730xl DNA Analyzer
- Beckmann CEQ™ 8000 and CEQ 8800 Genetic Analysis Systems

For further details about analysis of multiplex PCR products on high-resolution sequencing instruments, see Appendix C, page 43.

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. These include several manual and automatable products such as the QIAamp®, PAXgene® Blood DNA, and DNeasy® systems for rapid purification of human, plant, and animal genomic DNA as well as bacterial and viral nucleic acids. Alternatively, REPLI-g® Kits for whole genome amplification can be used. For more information about QIAamp, DNeasy, REPLI-g Kits, and the PAXgene Blood DNA System, contact one of our Technical Service Departments (see back cover) or visit the QIAGEN web site at www.qiagen.com.

Quantity of starting template

The quantity of starting template is also an important issue for successful multiplex PCR of mutation targets. For further detailed information of template quantity from different sources see Appendix B, page 38.

Choosing the correct protocol

This handbook contains 2 protocols.

Multiplex PCR of Fragments up to 1.5 kb in Length (page 16)

Choose this protocol — with optional use of Q-Solution (see page 8) — for multiplex PCR of fragments up to 1.5 kb, with subsequent analysis using agarose gels, the QIAxcel Advanced System, or the Agilent 2100 Bioanalyzer.

Multiplex PCR of Fragments up to 500 bp in Length (page 16)

Choose this protocol — with optional use of Q-Solution (see page 8) — for multiplex PCR of fragments smaller than 500 bp, with subsequent analysis using capillary sequencing instruments, agarose gels, the QIAxcel Advanced System, or the Agilent 2100 Bioanalyzer.

Special multiplex PCR applications

The QIAGEN Multiplex PCR *Plus* 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 semiquantitative gene expression analysis
- Exon-specific PCR

For further information on special multiplex PCR applications and optimization recommendations, see Appendices D and E on pages 44 and 45, respectively.

Protocol: Multiplex PCR of Fragments up to 1.5 kb in Length

This protocol is designed for amplification of target sequences up to 1.5 kb in length. Subsequent analysis may be performed using agarose gels, the QIAxcel Advanced System, or the Agilent 2100 Bioanalyzer. For longer PCR products than 1.5 kb, please see Appendix E, page 45. 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 E, page 45.

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 s.**
- **Use equal concentrations (0.2 µM) of all primers.**
- For optimal results, we recommend using primer pairs with a T_m of ≥68°C., see Appendix A, page 37 for multiplex PCR primer design.
- **Prepare a 10x primer mix as described in Table 2, page 12.**
- **Optional:** Q-Solution can be used for templates which are GC rich (>65%) or have a high degree of secondary structure. For further information on using Q-Solution, see page 8.
- 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.
- **Optional:** CoralLoad Dye can be used for easy visualization during PCR setup and subsequent detection of DNA migration distance during electrophoresis. Note that CoralLoad Dye must not be used on capillary sequencers.
- **PCR must start with an activation step of 5 min at 95°C** to activate HotStarTaq *Plus* DNA Polymerase (see step 5 of this protocol).

Procedure

1. **Thaw the 2x Multiplex PCR Master Mix (if stored at –20°C), template DNA, RNAse-free water, Q-Solution (optional), CoralLoad Dye (optional), and the 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 12).

2. Prepare a reaction mix according to Table 6.

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 the Multiplex PCR Master Mix to primer mix and template should be maintained as shown in Table 6.

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

Table 6. Reaction composition using 2x Multiplex PCR Master Mix, with optional use of Q-Solution and CoralLoad Dye

Component	Volume/reaction	Final concentration
Reaction mix 2x Multiplex PCR Master Mix*	25 µl	1x
10x primer mix, 2 µM each primer (see Table 2)	5 µl	0.2 µM†
Optional: Q-Solution, 5x	5 µl	0.5x
Optional: CoralLoad Dye, 10x‡	5 µl	1x CoralLoad Dye
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	≤300 ng DNA Start with 100 ng DNA
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 (i.e., 0.1–0.3 µM) may further improve amplification performance.

‡ If using capillary sequencers for detection, CoralLoad must not be used.

§ For volumes less than 50 µl, the 1:1 ratio of 2x Multiplex PCR Master Mix to primer mix and template 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 mixture 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 (≤300 ng /reaction) to the individual PCR tubes containing the reaction mix. See Table 6 for exact values.**
5. **a) Program the thermal cycler according to the manufacturer's instructions.**
   b) Place the PCR tubes in the thermal cycler and start the cycling program as outlined in Table 7.
   Each PCR program must start with an initial heat-activation step at 95°C for 5 min to activate HotStarTaq *Plus* DNA Polymerase.

Table 7. Optimized cycling protocol for multiplex PCR of up to 1.5 kb fragments, with optional use of Q-Solution and CoralLoad Dye

			Additional comments
Initial activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this step.
3-step cycling			
Denaturation:	30 s	95°C	
Annealing:	90 s	60°C	The annealing temperature of 60°C is suitable for most PCR systems. 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 1.5 kb in length. For targets longer than 1.5 kb, see Appendix E, page 45.
Number of cycles:	35		35 cycles give sufficient results in most cases. The number of cycles is dependent on the amount of template DNA and the required sensitivity of your detection method (see Table 8 for further recommendations).
Final extension:	10 min	68°C	

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

Table 8. Recommendations for template amount and cycle number

Amount of starting template (ng DNA per PCR reaction)*	Number of cycles
100–300	30–35
10–100	35–40
0.1–10	40–45

* Approximate value; for exact conversion rates see Appendix C, Table 15, page 42.

6. **After amplification, samples can be stored overnight at 2–8°C or at –20°C for long-term storage.**
7. **Analyze samples on an agarose gel or the QIAxcel Advanced System or the Agilent 2100 Bioanalyzer (see Tables 3, 4, and 5, pages 13 and 14 for further recommendations).**

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

Protocol: Multiplex PCR of Fragments up to 500 bp in Length

This protocol is designed for amplification of target sequences up to 0.5 kb in length. Subsequent analysis may be performed using capillary sequencers, agarose gels, the QIAxcel Advanced System, or the Agilent 2100 Bioanalyzer. 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 E, page 45.

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 s.**
- **Use equal concentrations (0.2 µM) of all primers.**
- For optimal results, we recommend using primer pairs with a T_m of ≥68°C., see Appendix A, page 37 for multiplex PCR primer design.
- **Prepare a 10x primer mix as described in Table 2, page 12.**
- **Optional**: Q-Solution can be used for templates which GC rich (>65%) or have a high degree of secondary structure. For further information on using Q-Solution, see page 8.
- 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.
- **Optional**: CoralLoad Dye can be used for easy visualization during PCR setup and subsequent detection of DNA migration distance during electrophoresis. Note that CoralLoad Dye must not be used on capillary sequencers.
- **PCR must start with an activation step of 5 minutes at 95°C** to activate HotStarTaq *Plus* DNA Polymerase (see step 5 of this protocol).
- If amplifying microsatellites, we recommend the dedicated Type-it® Microsatellite PCR Kit (cat. no. 206243).

Procedure

1. **Thaw the 2x Multiplex PCR Master Mix (if stored at –20°C), template DNA, RNase-free water, Q-solution (optional), CoralLoad Dye (optional), 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 12).

2. Prepare a reaction mix according to Table 9.

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 the Multiplex PCR Master Mix to primer mix and template should be maintained as shown in Table 9.

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

Table 9. Reaction composition using 2x Multiplex PCR Master Mix, with optional use of Q-Solution and CoralLoad Dye

Component	Volume/reaction	Final concentration
Reaction mix 2x Multiplex PCR Master Mix*	25 µl	1x
10x primer mix, 2 µM each primer (see Table 2)	5 µl	0.2 µM†
Optional: Q-Solution, 5x	5 µl	0.5x
Optional: CoralLoad Dye, 10x‡	5 µl	1x CoralLoad Dye
RNase-free water	Variable	–
Template DNA Template DNA, added at step 4	Variable	≤300 ng DNA; start with 100 ng DNA
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 (i.e., 0.1–0.3 µM) may further improve amplification performance.

‡ If using capillary sequencers for detection, CoralLoad must not be used.

§ For volumes less than 50 µl, the 1:1 ratio of 2x Multiplex PCR Master Mix to primer mix and template 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 (≤300 ng/reaction) to the individual PCR tubes containing the reaction mix. See Table 9 for exact values.**

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

   b) Place the PCR tubes in the thermal cycler and start the cycling program as outlined in Table 10, page 25.

   Each PCR program must start with an initial heat-activation step at 95°C for 5 min to activate HotStarTaq *Plus* DNA Polymerase.

Table 10. Optimized cycling protocol for multiplex PCR of up to 500 bp fragments, with optional use of Q-Solution and CoralLoad Dye

			Additional comments
Initial activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this step.
3-step cycling			
Denaturation:	30 s	95°C	
Annealing:	90 s	60°C	The annealing temperature of 60°C is suitable for most PCR systems. If the lowest T_m* of your primer mixture is below 60°C, use 57°C as starting annealing temperature.
Extension:	30 s	72°C	
Number of cycles:	35		35 cycles give sufficient results in most cases. The number of cycles is dependent on the amount of template DNA and the required sensitivity of your detection method (see Table 11, page 26, for further recommendations).
Final extension:	10 min	68°C	For analysis on capillary sequencers, a final extension time of 30 min must be used.†

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

† Allows generation of A-overhangs by HotStarTaq *Plus* DNA Polymerase that are required for high-resolution analysis using capillary- or gel-based DNA sequencers.

Table 11. Recommendations for template amount and cycle number

Amount of starting template (ng DNA per PCR reaction)*	Number of cycles†
100–300	30–35
10–100	35–40
0.1–10	40–45

* Approximate value; for exact conversion rates see Appendix B, Table 15, page 42.

† If using fluorescently labeled primers and a capillary sequencing instrument as analysis platforms, in general 5 cycles less are recommended.

6. **After amplification, samples can be stored overnight at 2–8°C or at –20°C for long-term storage.**
7. **Analyze samples on a capillary sequencer, an agarose gel or the QIAxcel System or the Agilent 2100 Bioanalyzer (Tables 3, 4, and 5, pages 13 and 14, for further recommendations).**

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

Troubleshooting Guide

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

	Comments and suggestions
Little or no product	
a) HotStarTaq *Plus* DNA Polymerase not activated	Ensure that the cycling program included the HotStarTaq *Plus* DNA Polymerase activation step (5 min at 95°C) as described in step 5 of the protocols (pages 19 and 24).
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 many targets in parallel, a primer concentration of 0.1 µM and extension time of 3 min may improve results. We do not recommend using primer concentrations higher than 0.3–0.4 µM for multiplex PCR followed by agarose-gel-based or QIAxcel-based detection, as this may affect multiplex PCR fidelity. For multiplex PCR followed by sequencer-based fragment analysis, a primer concentration of 1–2 µM (only for the primers generating weak signals) and an extension time of 3 min may improve results. Check the concentration of primer stock solutions. For calculation of primer concentration, refer to Table 2, page 12.
d) Insufficient number of cycles	Increase number of PCR cycles. Refer to Table 8 (page 20) and Table 11 (page 26), for guidelines.

	Comments and suggestions
e) PCR cycling conditions not optimal	Check that the correct cycling conditions were used (see Tables 7 and 10 on pages 20 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 E, page 45).
f) PCR cycling conditions not optimal	Check functionality and specificity of primer pairs in singleplex reactions. Ensure that primers of sufficiently high quality were used. For detection on capillary sequencing instruments, ensure that the primers are labeled with fluorescent dyes. 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.
g) Annealing temperature too high	Follow the recommendations given in Appendix B, page 38 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 E, page 45) 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. 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 37 for general guidelines on multiplex PCR primer design.

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

	Comments and suggestions
i) Insufficient starting template	Increase amount of starting template up to 300 ng per 50 µl reaction for gel-based detection and up to 200 ng per 50 µl reaction for sequencer-based detection.
k) Low-quality starting template	Use only high-quality DNA, such as that purified using DNeasy Kits.
l) Problems with starting template	Check the concentration, storage conditions, and quality of the starting template (see Appendix B, page 38). If necessary, make new serial dilutions of template nucleic acid from stock solutions. Repeat the multiplex PCR using the new dilutions
m) PCR product is too long	The optimized protocols allow amplification of target sequences up to 1.5 kb. For longer PCR products, see Appendix E, page 45.
n) 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.
o) Problems with the thermal cycler	Check the power to the thermal cycler and that the thermal cycler has been correctly programmed.
p) No final extension step, or final extension step was not optimal	Ensure that the final extension step was performed as described in (see Tables 7 and 10 on pages 20 and 25, respectively). For sequencer-based analysis, a final extension step of 30 min at 60°C should be used. If necessary, it can be prolonged to 45 min. When detecting PCR products on agarose gels, the QIAxcel Advanced System, or the Agilent 2100 Bioanalyzer, 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.

Comments and suggestions	
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 many targets (≥10) in parallel followed by detection on sequencing instruments, a primer concentration of 1–2 µM only for the primers generating weak signals and an extension time of 3 min may improve results. We do not recommend using primer concentrations higher than 0.3–0.4 µM for detection on QIAxcel Advanced or agarose gels, as this may affect multiplex PCR fidelity. Check concentration of primer stock solutions (see Appendix B, page 38).
c) PCR cycling conditions not optimal	Check that the correct cycling conditions were used (see Tables 7 and 10 on pages 20 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 E, page 45).

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

	Comments and suggestions
d) No final extension step, or final extension step was not optimal	Ensure that the final extension step was performed as described in (see Tables 7 and 10 on pages 20 and 25, respectively). For sequencer-based analysis, a final extension step of 30 min at 60°C should be used. If necessary, it can be prolonged to 45 min. When detecting PCR products on agarose gels, the QIAxcel Advanced System, or the Agilent 2100 Bioanalyzer, 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.
e) Annealing temperature too high	Check that the correct cycling conditions were used (see Tables 7 and 10 on pages 20 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 E, page 45).
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. 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.
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 7 and 10 on pages 20 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 E, page 45).

	Comments and suggestions
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 for gel-based detection and 1–2 cycles for sequencer-based detection.
c) Annealing temperature too low	Follow the recommendations given in Appendix B, page 38, 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 Appendix E, page 45).
d) Mg^{2+} concentration not optimal	Use an initial Mg^{2+} concentration of 3 mM as provided by the 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 many targets (≥10) in parallel followed by detection on sequencing instruments, a primer concentration of 1–2 µM only for the primers generating weak signals and an extension time of 3 min may improve results. We do not recommend using primer concentrations higher than 0.3–0.4 µM for detection on the QIAxcel Advanced or agarose gels, as this may affect multiplex PCR fidelity. Check the concentration of primer stock solutions (see Appendix B, page 38).
f) Primer design not optimal	Review primer design. Refer to Appendix A, page 37, 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.

	Comments and suggestions
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 sequences	Primers 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 37 for general guidelines on multiplex PCR primer design.
j) GC-rich template or template with a high degree of secondary structure	Using the same cycling conditions, repeat the multiplex PCR using Q-Solution. 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 Appendix B, Table 16, page 38). Repeat the multiplex PCR using less DNA (i.e., <300 ng 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 safety data sheets (SDSs), available from the product supplier.

	Comments and suggestions
c) No final extension step, or final extension step was not optimal	Ensure that the final extension step was performed as described in Tables 7 and 10 on pages 20 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.
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.

Optimization of PCR conditions for analysis on capillary or gel-based sequencers

Additional products are observed

a) Amount of sample loaded is too high	Loading of large amounts of PCR product may result in additional peaks. Decrease the cycle number and/or the template amount in the PCR reaction until the background is decreased to a satisfactory level with acceptable peak heights (e.g., typical peak heights <10000 relative fluorescent units on ABI 3730 or 3730xl DNA Analyzer).
b) Faint peaks ("stutter peaks")	Amplification of some 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 cycle number to reduce this effect. If the length of the faint peak is one base shorter than the main peak, refer to "n–1 products detected" below.

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

	Comments and suggestions
c) Sample not completely denatured	Denature the samples before loading by heating to 95°C for 5 min. Deionized formamide should be preferred compared to water.
d) n–1 products detected	Ensure that the final extension step was performed as described in Tables 7 and 10 on pages 20 and 25, respectively. The final extension step can be increased to 45 min to improve results. If the final extension step was correctly performed, decrease the number of cycles and/or template amount.
e) Differing signal intensities	Different fluorescent dyes may give differing signal intensities on a particular detection instrument although comparable amounts of PCR product were added. We recommend combining fluorescent dyes for multiplex PCR according to the instructions of the detection instrument's manufacturer.
Some products are missing in a multiplex experiment	
a) Amount of template loaded is too low	Loading of small amounts of PCR product may result in the dropout of some peaks after sequencer analysis. Increase number of cycles by an increment of 1–2 cycles until all products are in the range of signals specified by the instrument manufacturer.

	Comments and suggestions
b) Uneven amplification of different products	The signal of weak peaks obtained when performing fragment analysis on sequencing instruments can be improved by increasing the cycle number and decreasing the template amount during PCR. An annealing time of 3 min instead of 90 s can also help to increase weak signals compared to the highest peaks in a multiplex fragment analysis. If the signals of some peaks are still too low, increase the primer concentration only of the primer pairs generating weak signals. An increase to 1 µM for up to 10 amplicons and to 2 µM for more than 10 amplicons is recommended. Some primers pairs may result in lower signals than others. Check whether your primers were designed according to the recommendations In Appendix B, page 38. If not, redesign your primers. Alternatively try to use Q-Solution to improve the amplification of the weak primer pairs.
Faint peaks or no allele peaks	
a) Poor capillary electrophoresis (size standard also affected)	Inject the sample again. Check the syringe O-ring for injection leakage. Check that the fluorescence detection instrument is functioning correctly.
b) Poor quality formamide	Use high-quality formamide for the analysis of samples used on capillary sequencing instruments. The conductivity of the formamide should be <100 µS/cm
Broad peaks; peaks get smaller towards the end of the analysis	
Sample not completely denatured	Use deionized formamide for diluting the samples before injecting into a sequencing instrument. Samples are more stable in formamide than in water. Perform a denaturation step of 5 min at 95°C before loading.

Appendix A: Design of Multiplex 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 combining them in a multiplex PCR assay.

Annealing temperature

If necessary, perform a gradient PCR to determine the optimal annealing temperature (see Appendix E, page 45). Otherwise, use the recommendations in Table 12.

Table 12. 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.
- 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://frodo.wi.mit.edu/primer3/) 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, 4, and 5, pages 13 and 14), 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

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.

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

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} (OD) = ε_{260} x 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:

A_{260} (OD)= 0.89 x [(*n*A x 15,480) + (*n*C x 7340) + (*n*G x 11,760) + (*n*T x 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

ε_{260} = 0.89 x [(6 x 15,480) + (6 x 7340) + (6 x 11,760) + (6 x 8850)] = 231,916

Concentration = A_{260} (OD)/ε_{260} = 0.283/231,916 = 1.22 x 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 12).

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.

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 12).

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

Template quality

Template quality is of extreme importance. Impurities have inhibitory effects on PCR. These are listed in Table 13.

Visit www.qiagen.com to see our complete range of DNA purification products, all of which provide pure DNA from a wide variety of sample types and are ideal for accurate PCR results.

Table 13. Impurities exhibiting inhibitory effects on PCR

Impurity	Inhibitor concentration
SDS	> 0.005% (w/v)
Phenol	> 0.2% (v/v)
Ethanol	> 1% (v/v)
Isopropanol	> 1% (v/v)
Sodium acetate	5 mM
Sodium chloride	25 mM
EDTA	0.5 mM
Hemoglobin	1 mg/ml
Heparin	0.15 i.U./ml
Urea	> 20 mM
RT reaction mixture	15% (v/v)

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 14 and 15, respectively. The QIAGEN Multiplex PCR *Plus* Kit enables successful multiplex amplification using a wide range of template amounts down to the picogram level.

Table 14. 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

Table 15. 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.

Table 16. Conversion of copy numbers of starting template for different DNA sources

Number of copies of starting template	1 kb DNA	*E. coli* DNA*	Human genomic DNA
100–1000	0.11–1.1 fg	0.56–5.56 pg	0.36–3.6 ng
>1 x 10^3 – 5 x 10^4	1.1–55 fg	5.56–278 pg	3.6–179 ng
>5 x 10^4	>55 fg	>278 pg	>179 ng

* Refers to single-copy genes.

Appendix C: Detection of Targets Amplified with the QIAGEN Multiplex PCR *Plus* Kit Using Fluorescently Labeled Primers

Fluorescent labels

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.

Guidelines for analysis of targets on capillary sequencers

Choose an appropriate PCR cycle number and template amount for PCR to avoid excessively high signals or remnants of the PCR product in the capillaries. If signals are too high, use less template or fewer PCR cycles to bring signal in the recommended signal intensity range as recommended by the instrument manufacturer.

For analysis of mutation targets amplified with QIAGEN Multiplex PCR *Plus* Kit using high-resolution sequencing instruments, like Applied Biosystems 3730 or 3730xl Genetic Analyzer, primers as well as the size standard must be labeled using compatible fluorescent dyes. Samples are mixed with the size standard, diluted, and heat denatured before analysis on a capillary sequencer. See Table 17 for further information about how to prepare samples.

Note: Overloading of the sample may harm your sequencing instrument. If high signal intensities are obtained (for example, when using template amounts that are >10 ng per PCR reaction and/or when having >28 cycles), desalting of PCR products before loading on the capillary sequencer using, for example, DyeEx® Kits (DyeEx 96 Kit [4], cat no. 63181) may increase the life span of the capillaries. Avoid empty wells when performing fragment analysis on capillary sequencers to protect the capillaries from running dry; fill empty wells with deionized water.

Table 17. Sample handling for fragment analysis of PCR products on capillary sequencing instruments

Step	Range	Start with
Sample preparation	Prepare DNA e.g., by using QIAamp or DNeasy Kits	QIAamp
Amount of DNA	0.1–200 ng/reaction	10 ng
Sample dilution	1:10 –1:50 in deionized formamide or water	1:10 in deionized formamide
Size of the ladder	Add a labeled size standard for sizing of fragment analysis samples. For example GeneScan™ 500 LIZ® Size Standard (ABI) Part Number: 4322682 Add 0.2–0.5 µl/reaction	0.3 µl/reaction
Denaturation	Heat for 3–5 min at 95°C	5 min at 95°C
Time until loading	Load within 1 hour	

Appendix D: Special Multiplex PCR Applications

Sensitive multiplex PCR mutation 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 *Plus* DNA Polymerase and the Multiplex PCR *Plus* Buffer increases specificity both at the start of and during PCR, making the QIAGEN Multiplex PCR *Plus* Kit well-suited for highly sensitive mutation assays such as detecting small amounts of a mutated cancer gene within a range of healthy cells. 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.

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. The QIAGEN Multiplex PCR *Plus* Kit 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.

Appendix E: Optimization of PCR Conditions for Special Multiplex PCR Applications

QIAGEN Multiplex PCR *Plus* 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.

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 37, 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.

Large number of PCR products

For multiplex amplification reactions with more than 10 PCR products, an increase of the annealing time from 90 seconds to 3 minutes can improve product yield. 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 Appendix B, page 38). The use of high-quality (e.g., HPLC purified) primers is recommended, although standard quality may also be sufficient. When using fluorescently labelled primers, the use of high-quality primers (e.g., HPLC grade) is recommended. We strongly recommend only combining primers of comparable quality.

Highly sensitive mutation detection or low template amounts

Increasing annealing time from 90 seconds to 3 minutes may further increase sensitivity (see section above).

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 Appendix B, page 38). Differences in primer concentration due to incorrect quantification or dilution are the most likely cause of nonuniform product yield.

The signal of weak peaks obtained when performing fragment analysis on sequencing instruments can be improved by increasing the cycle number and decreasing the template amount during PCR. An annealing time of 3 min instead of 90 s can also help to increase weak signals compared to the highest peaks in a multiplex fragment analysis. If the signals of some peaks are still too low, increase the primer concentration only of the primer pairs generating weak signals. An increase to 1 µM for up to 10 amplicons and to 2 µM for more than 10 amplicons is recommended.

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 45), 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.

Ordering Information

Product	Contents	Cat. no.
QIAGEN Multiplex PCR *Plus* Kit (30)	For 30 x 50 µl multiplex PCR reactions: 2x Multiplex PCR Master Mix (1 x 0.85 ml), 5x Q-Solution (1 x 2 ml), RNase-Free water (1 x 1.9 ml), 10x CoralLoad Dye (1 x 1.2 ml)	206151
QIAGEN Multiplex PCR *Plus* Kit (100)	For 100 x 50 µl multiplex PCR reactions: 2x Multiplex PCR Master Mix (3 x 0.85 ml), 5x Q-Solution (1 x 2 ml), RNase-Free water (2 x 1.9 ml), 10x CoralLoad Dye (1 x 1.2 ml)	206152
QIAGEN Multiplex PCR *Plus* Kit (1000)	For 1000 x 50 µl multiplex PCR reactions: 2x Multiplex PCR Master Mix (2 x 25 ml), 5x Q-Solution (1 x 10 ml), RNase-Free water (1 x 20 ml), 10x CoralLoad Dye (1 x 5.5 ml)	206155
Related Products		
Type-it Microsatellite PCR Kit — for reliable microsatellite analysis by multiplex PCR		
Type-it Microsatellite PCR Kit (70)*	For 70 x 25 µl reactions: Type-it Multiplex PCR Master Mix,† 5x Q-Solution, and RNase-Free Water	206241
Type-it Mutation Detect PCR Kit — for reliable mutation detection by multiplex PCR		
Type-it Mutation Detect PCR Kit (70)*	For 70 x 25 µl reactions: Type-it Multiplex PCR Master Mix,† 5x Q-Solution, RNase-Free Water, and CoralLoad Dye	206341
HotStarTaq *Plus* DNA Polymerase — for highly specific hot-start PCR		
HotStarTaq *Plus* DNA Polymerase (250 U)*	250 units HotStarTaq *Plus* DNA Polymerase, 10x PCR Buffer,‡ 10x CoralLoad PCR Buffer, 5x Q-Solution, 25 mM $MgCl_2$	203603

* Other kit sizes/formats available; see www.qiagen.com.

† Master Mix contains HotStarTaq *Plus* DNA Polymerase, optimized $MgCl_2$ concentration, and 200 µM each dNTP.

‡ Contains 15 mM $MgCl_2$.

Product	Contents	Cat. no.
HotStarTaq *Plus* Master Mix Kit — for fast and highly specific amplification		
HotStarTaq *Plus* Master Mix Kit (250)*	3 x 0.85 ml HotStarTaq *Plus* Master Mix,† containing 250 units of HotStarTaq *Plus* DNA Polymerase total, 1 x 0.55 ml CoralLoad Concentrate, 2 x 1.9 ml RNase-Free Water for 250 x 20 µl reactions	203643
HotStar HiFidelity Polymerase Kit — for highly sensitive and reliable high-fidelity hot-start PCR		
HotStar HiFidelity Polymerase Kit (100 U)*	100 units HotStar HiFidelity DNA Polymerase, 5x HotStar HiFidelity PCR Buffer (inc. dNTPs),‡ 5x Q-Solution, 25 mM $MgSO_4$, RNase-Free Water	202602
QIAGEN LongRange PCR Kit — for reliable amplification of long fragments up to 40 kb in length		
QIAGEN LongRange PCR Kit (20)*	For 20 x 50 µl reactions: LongRange PCR Enzyme Mix (40 U), LongRange PCR Buffer, 5x Q-Solution, RNase-Free Water, 10 mM dNTPs	206401
QIAamp DNA Kits — for genomic, mitochondrial, bacterial, parasite, or viral DNA		
QIAamp DNA Mini Kit (50)*	For 50 DNA preps: 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, Reagents, Buffers, Collection Tubes (2 ml)	51304
DNeasy Blood & Tissue Kits — for purification of total DNA from animal blood and tissues, and from cells, yeast, bacteria, or viruses		
DNeasy Blood & Tissue Kit (50)*	50 DNeasy Mini Spin Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	69504

* Other kit sizes/formats available; see www.qiagen.com.

† Contains 3 mM $MgCl_2$ and 400 µM each dNTP.

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

Product	Contents	Cat. no.
dNTP Set and dNTP Mix, PCR Grade — for sensitive and reproducible PCR and RT-PCR		
dNTP Mix, PCR Grade (200 µl)*	Mix containing 10 mM each of dATP, dCTP, dGTP, and dTTP (1 x 200 µl)	201900
dNTP Set, PCR Grade, 4 x 100 µl*	100 mM each dATP, dCTP, dGTP, dTTP for 1000 x 50 µl PCR reactions	201912

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

Notes

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