December 2009

Type-it® Mutation Detect PCR Handbook

For multiplex PCR-based mutation detection or preamplification of SNPs, without the need for optimization

QIAGEN®

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.

QIAGEN sets standards in:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

Our mission is to enable you to achieve outstanding success and breakthroughs. For more information, visit www.qiagen.com.

Contents

Kit Contents

Type-it Mutation Detect PCR Kit	**(70)**	**(200)**
Catalog no.	**206341**	**206343**
Number of reactions	**70**	**200**
Type-it Multiplex PCR Master Mix, 2x*	1 x 0.85 ml	3 x 0.85 ml
Q-Solution®, 5x	1 x 2 ml	1 x 2 ml
RNase-Free Water	1 x 1.9 ml	2 x 1.9 ml
CoralLoad® Dye, 10x	1 x 1.2 ml	1 x 1.2 ml
Handbook	1	1

* Contains HotStarTaq® *Plus* DNA Polymerase, Type-it Mutation Detect PCR Buffer with optimized concentration of $MgCl_2$, and dNTPs.

Shipping and Storage

The Type-it Mutation Detect PCR Kit is shipped on dry ice. It should be stored immediately upon receipt at –20°C in a constant-temperature freezer. When stored under these conditions and handled correctly, the product can be kept at least until the expiration date (see the inside of the kit lid) without showing any reduction in performance. The 2x Type-it Multiplex PCR Master Mix can be stored at 2–8°C for up to 2 months without showing any reduction in performance.

Product Use Limitations

The Type-it Mutation Detect PCR Kit is intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

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

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

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 sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the Type-it Mutation Detect 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/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Quality Control

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

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/support/MSDS.aspx where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Product Specifications

2x Type-it 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:

Type-it Mutation Detect 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
CoralLoad Dye:	10x concentrated
RNase-free water:	Ultrapure quality, PCR-grade

Introduction

Accurate genotyping analysis often requires extensive optimization of experimental parameters. Sample materials may be limiting in genotyping studies, for example, when large numbers of SNPs need to be analyzed or when working with sample materials such as biopsies or formalin-fixed, paraffin embedded (FFPE) tissue. Some studies require analysis of a large number of different mutations of a certain gene related to a disease (e.g., deletions, translocations, or SNPs). Including the necessary internal controls, a large number of PCR reactions are required when performing singleplex- or lowplex-grade PCR analysis, leading to increases in both costs and analysis time. QIAGEN recognizes these challenges and has developed the Type-it PCR Kits — a new PCR-based product line dedicated for different genotyping applications, ranging from analysis of single nucleotide polymorphisms (SNPs) to detection of mutations and identification of microsatellite loci.

The Type-it Mutation Detect PCR Kit is specifically developed and functionally validated for multiplex PCR-based analysis of mutations such as deletions or insertions and detection of genetically modified organisms or microbes. The kit is also suitable for use as a preamplification method for commercially available SNP detection PCR-based systems such as the SNaPshot™ system provided by Applied Biosystems.

This handbook contains dedicated protocols for detection of mutations. The Type-it Mutation Detect PCR Kit is available in an optimized, ready-to-use master mix format, simplifying multiplex PCR assay development.

Mutation detection

Mutations are changes in the genome of an organism that have not been caused by recombination and that are related to changes in the genetic information. This might also lead to a changed phenotype. Mutations can change the DNA sequence or the number or the order of the chromosomes of an individual and can be related to diseases or other clinically relevant events.

Targeted mutations such as knock-out mutations, for example in mice, are used as tools to study the function of certain genes and to investigate biological mechanisms and are an important basis of pharmacological research.

Genetically modified or transgenic organisms also include changes of certain gene loci by carrying genetic information derived from other species. Transgenic microorganisms are used routinely for the large-scale production of antibiotics, insulin, or vitamins.

Mutations can be classified as gene mutations, chromosomal mutations, and genomic mutations. Mutations within genes and chromosomes include insertions, deletions, duplications, translocations, and point mutations (see Table 1).

Table 1. Different types of mutations

Type of mutation	Description
Substitution	One base is exchanged for another
Deletion	Missing part of a chromosome or gene
Translocation	A chromosome breaks and a part is transferred to another chromosome
Duplication	Part of a chromosome or gene is duplicated
Inversion	After a double break of a chromosome, a part is inserted again in the opposite direction
Insertion	Additional insert within a chromosome or gene
Point mutation	Change of one single base
Knock-out	One or several genes are inactivated through experimental intervention
Transgene	Organism carrying genetic information derived from another species or organism

Mutations are analyzed to investigate changes in the genome of an individual due to genetic disorders, environmental influences (such as radiation), or disease-related phenotypes. The analysis of mutations requires highly specific and sensitive detection of the mutated locus and a comparison with the wild-type locus. Additionally, it is useful to be able to detect the mutated and the wild-type locus in parallel or to be able to detect several different mutations in the same context simultaneously. For this purpose, reliable multiplex PCR-based technology, as provided by the Type-it Mutation Detect PCR Kit, is required.

Polymorphisms

Polymorphisms are naturally occurring genetic differences in a population or between individuals. These differences occur with a higher frequency than typical mutations. Polymorphisms are scientifically relevant due to their high frequency and high variability. They are used for the identification of individuals and relationships as well as to investigate individual differences related to drug metabolism.

SNP analysis

SNPs are single nucleotide changes occurring at specific points within the genome. Analysis of SNPs is important, because they are genetic markers that can be used to associate genetic changes with disease or other alterations in phenotype. The study of SNPs can also be used to analyze complex phenotypic changes or to investigate whether a genetic relationship exists between certain individuals. To provide sufficient material for analysis, the regions carrying the relevant SNPs can be preamplified from genomic DNA by multiplex PCR, using the Type-it Mutation Detect PCR Kit, and can be used in subsequent SNP analysis (e.g., using SNaPshot technology).

Fast and simple method for reliable detection of mutations

The Type-it Mutation Detect PCR Kit is provided in a ready-to-use master mix format based on proven QIAGEN Multiplex Technology (patent pending). The master mix contains optimized concentrations of HotStarTaq *Plus* DNA Polymerase, $MgCl_2$ and dNTPs, and an innovative PCR buffer, specially developed for multiplex PCR-based detection of mutations or for preamplification of genomic SNP loci (see Table 2). It also includes the novel additive Factor MP, and a balanced combination of salts and additives, which enable comparable efficiencies for annealing and extension of all primers in the reaction (see Figure 1). Dedicated, application-specific protocols optimized for simultaneous amplification and subsequent detection of loci carrying mutations or SNPs are included to ensure reliable results for routine analysis or establishment of new assays.

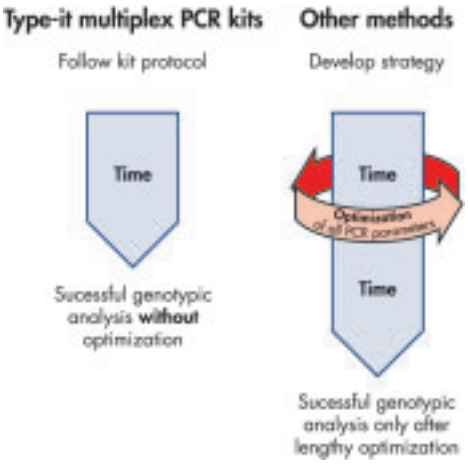

Figure 1. Successful multiplex PCR-based genotypic analysis without the need for optimization. The Type-it Mutation Detect PCR Kit is based on patent-pending QIAGEN Multiplex Technology and provides a simple procedure for reliable genotyping results. In contrast to current methods, the need for optimization of PCR parameters such as Mg^{2+} concentration and cycling conditions is eliminated.

Table 2. Applications of the Type-it Mutation Detect PCR Kit

Dedicated applications	Fields of research
Deletions	
Insertions	Typing of disease loci
Duplications	GMO analysis
Translocations	Typing of transgenic plants or animals
SNP preamplification (SNaPshot Multiplex Kit)	

HotStarTaq *Plus* DNA Polymerase

The Type-it 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.

Type-it Mutation Detect PCR Buffer

The unique Type-it Mutation Detect PCR Buffer facilitates the amplification of multiple PCR products. Some studies require analysis of a large number of different mutations of a certain gene related to a disease (e.g., deletions, translocations, or SNPs). Including the necessary internal controls, a large number of PCR reactions are required when performing singleplex- or lowplex-grade PCR analysis. The Type-it Mutation Detect PCR Buffer ensures comparable amplification efficiency for all amplicons in a highplex-grade multiplex experiment and allows more mutation targets to be combined without the loss of amplification efficiency for any of the targets.

In contrast to conventional PCR reagents, the Type-it Mutation Detect PCR Buffer contains a specially developed, balanced combination of salts and additives to ensure comparable efficiencies for annealing and extension of all primers in the reaction. Commonly employed optimization procedures for multiplex PCR are virtually eliminated. 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 Type-it Mutation Detect 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 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. For further information, please read the recommendations about Q-Solution, Appendix A, page 37.

CoralLoad Dye, 10x

The Type-it Mutation Detect PCR 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. When using CoralLoad Dye in the multiplex PCR reaction, the amplicons can be directly loaded onto an agarose gel, without prior addition of loading buffer. CoralLoad Dyes do not interfere with the QIAxcel® System and 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.

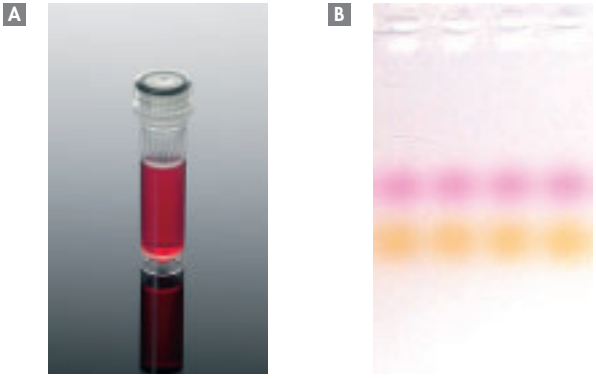

Figure 2. CoralLoad PCR Buffer and Concentrate. CoralLoad PCR Buffer and Concentrate A contain gel-tracking dyes B enabling immediate loading of PCR products and easy visualization of DNA migration.

Equipment and Reagents to Be Supplied by User

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

- Reaction tubes
- Pipets and pipet tips (aerosol resistant)
- Thermal cycler
- 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 Type-it Mutation Detect PCR Kit can be used with standard-quality primers 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 3, page 14). 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 primers labeled with fluorescent dyes 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 C and Appendix D, pages 40 and 45, 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 3 (page 14) 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 3. 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

The detection of mutation targets amplified by the Type-it Mutation Detect PCR Kit can be easily performed on various detection platforms.

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

Analysis of mutations on high-resolution sequencing instruments requires primers labeled with fluorescent dyes and allows resolution down to single bases.

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 Type-it Mutation Detect PCR Kit with different detection systems are provided in Tables 4, 5, and 6 (pages 15 and 16).

Table 4. 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%

* Cycling protocol is designed for amplicons up to 500 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 5. Analysis of multiplex PCR products using the QIAxcel 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

‡ The QX DNA High Resolution Cartridge is the recommended cartridge for analysis of multiplex PCR products obtained with the Type-it Mutation Detect PCR Kit.

§ Cycling protocols are designed for amplicons up to 500 bp in length. For longer targets, up to 1.5 kb in size, please refer to the relevant recommendations in the protocol.

Table 6. 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%

* This protocol is mainly designed for amplicons up to 500 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 Type-it Mutation Detect PCR 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
- Beckman CEQ™ 8000 and CEQ 8800 Genetic Analysis Systems

For further details about analysis of multiplex PCR products on high-resolution sequencing instruments, see Appendix D, page 45.

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, amplify very small amounts of genomic DNA while introducing no sequence bias, 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 C, page 40.

Choosing the correct protocol

This handbook contains 2 protocols.

Amplification of Mutations (Detection on Agarose Gels or the QIAxcel System or Agilent 2100 Bioanalyzer) (page 18)

Choose this protocol for multiplex PCR amplification of mutations with subsequent analysis using agarose gels or the QIAxcel System or the Agilent 2100 Bioanalyzer system. This protocol is also recommended for preamplification of SNPs if using SNaPshot Technology. **The protocol is suitable for medium- to low-resolution detection of amplicons using nonfluorescent primers.**

Amplification of Mutations (Detection on Capillary or Gel-Based Sequencing Instruments) (page 23)

Choose this protocol for multiplex PCR amplification of mutations using primers that are labeled with fluorescent dyes with subsequent analysis using a capillary or gel-based sequencing instrument. Protocol contains optimized cycle number and final extension step for fluorescence detection. **The protocol is suitable for detection of amplicons with high resolution (down to 1 bp) using fluorescent primers.**

Protocol: Amplification of Mutations (Detection on Agarose Gels or the QIAxcel System or Agilent 2100 Bioanalyzer)

This protocol is suitable for medium- to low-resolution detection of amplicons using nonfluorescent primers.

Important points before starting

- **This protocol is also recommended for preamplification of SNPs when using SNaPshot technology.**
- **Always start with the cycling conditions specified in this protocol.**
- If using an already established multiplex PCR system for the detection of mutations, 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 B, page 38 for multiplex PCR primer design.
- **Prepare a 10x primer mix as described in Table 3, page 14.**
- When using Q-Solution, follow the recommendations in Appendix A, page 37. In your first attempt, start without using Q-Solution.
- **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 6 of this protocol).

Procedure

1. **Thaw the 2x Type-it Multiplex PCR Master Mix (if stored at –20°C), template DNA, RNAse-free water, CoralLoad Dye (optional), Q-Solution (optional), and the primer mix. Mix the solutions completely before use.**

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

2. **Prepare a reaction mix according to Table 7. When using Q-Solution, follow Table 8.**

   Note: 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 25 µl, the 1:1 ratio of Type-it Multiplex PCR Master Mix to primer mix and template should be maintained as shown in Tables 7 and 8

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

Table 7. Reaction composition using 2x Type-it Multiplex PCR Master Mix without Q-Solution

Component	Volume/reaction	Final concentration
Reaction mix	12.5 µl	1x
2x Type-it Multiplex PCR Master Mix*		
10x primer mix, 2 µM each primer (see Table 3)	2.5 µl	0.2 µM†
Optional: CoralLoad Dye, 10x‡	2.5 µl	1x
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	≤300 ng DNA Start with 100 ng DNA
Total volume	25 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 Dye must not be used.

§ For volumes less than 25 µl, the 1:1 ratio of 2x Type it Multiplex PCR Master Mix to primer mix and template should be maintained.

Table 8. Reaction composition using 2x Type-it Multiplex PCR Master Mix with Q-Solution

Component	Volume/reaction	Final concentration
Reaction mix	12.5 µl	1x
2x Type-it Multiplex PCR Master Mix*		
10x primer mix, 2 µM each primer (see Table 3)	2.5 µl	0.2 µM†
Optional: CoralLoad Dye, 10x‡	2.5 µl	1x
Q-Solution, 5x	2.5 µl	0.5x
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	≤300 ng DNA Start with 100 ng DNA
Total volume	25 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 Dye must not be used.

§ For volumes less than 25 µl, the 1:1 ratio of 2x Type-it 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.**

   Note: 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 or wells containing the reaction mix. See Table 10, page 21 for exact values.**

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

6. **Place the PCR tubes or plate in the thermal cycler and start the cycling program as outlined in Table 9, page 21.**

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

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

Table 9. Optimized cycling protocol for mutation detection PCR using 2x Type-it Multiplex PCR Master Mix with or without Q-Solution

Step	Time	Temp.	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 the starting annealing temperature.
Extension:	30 s	72°C	Optimal for targets up to 0.5 kb in length. For targets longer than 0.5 kb, increase the extension time by 30 s per 0.5 kb.
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 10 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 10. Recommendations for template amount and cycle number

Amount of starting template (ng DNA per 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 18, page 44.

7. **Analyze samples on an agarose gel or the QIAxcel System or the Agilent 2100 Bioanalyzer (see Tables 4, 5, and 6, pages 15 and 16, 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: Amplification of Mutations (Detection on Capillary or Gel-Based Sequencing Instruments)

This protocol is suitable for detection of amplicons with high resolution down to 1 bp using fluorescent primers.

Important points before starting

- **Always start with the cycling conditions specified in this protocol.**
- If using an already established multiplex PCR system for mutation detection, 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 B, page 38 for multiplex PCR primer design.
- **Prepare a 10x primer mix as described in Table 3, page 14.**
- When using Q-Solution, follow the recommendations in Appendix A (page 37). In your first approach, start without Q-Solution.
- **Do not use CoralLoad Dye before subsequent detection on capillary or gel-based sequencing instruments as it might interfere with the detection method.**
- PCR must start with **an activation step of 5 min at 95°C** to activate HotStarTaq *Plus* DNA Polymerase (see step 6 of this protocol).

Procedure

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

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

2. **Prepare a reaction mix according to Table 11.**

   Note: 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 25 µl, the 1:1 ratio of Type-it Multiplex PCR Master Mix to primer mix and template should be maintained as shown in Table 11.

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

Table 11. Reaction composition using 2x Type-it Multiplex PCR Master Mix

Component	Volume/reaction	Final concentration
Reaction mix	12.5 µl	1x
2x Type-it Multiplex PCR Master Mix*		
10x primer mix, 2 µM each primer (see Table 3)	2.5 µl	0.2 µM†
Optional: Q-Solution, 5x	2.5 µl	0.5x
Note: Do not use CoralLoad Dye when analyzing PCR products on capillary sequencers		
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	≤200 ng DNA; start with 10 ng DNA
Total volume	25 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.

‡ For volumes less than 25 µl, the 1:1 ratio of 2x Type-it 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.**

   Note: 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 (≤200 ng/reaction) to the individual PCR tubes or wells containing the reaction mix. See Table 13, page 25 for exact values.**

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

6. **Place the PCR tubes or plate in the thermal cycler and start the cycling program as outlined in Tables 12, page 25.**

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

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

Table 12. Optimized cycling protocol for multiplex PCR amplification of mutations

Step	Time	Temp.	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 the starting annealing temperature.
Extension:	30 s	72°C	Optimal for targets up to 0.5 kb in length. For targets longer than 0.5 kb, increase the extension time by 30 s per 0.5 kb.
Number of cycles:	28		28 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 13 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]).

† For targets longer than 0.5 kb, increase the extension time by 30 s per 0.5 kb.

Table 13. Recommendations for template amount and cycle number

Amount of starting template (ng DNA per PCR reaction)‡	Number of cycles
50–200	20–24
10–50	24–28
0.1–10	28–32

‡ Approximate value; for exact conversion rates see Appendix C, Table 18, page 43.

7. **Prepare a dilution of the PCR products and analyze samples on a high-resolution capillary sequencing instrument (see page 16 and Appendix D, page 45, 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 6 of the protocols (pages 21 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 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 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 3, page 14.
d)	Insufficient number of cycles	Increase the number of PCR cycles. Refer to Table 9 (page 21) and Table 12 (page 25), for guidelines.

		Comments and suggestions
e)	PCR cycling conditions not optimal	Check that the correct cycling conditions were used (see Tables 9 and 12 on pages 21 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 F, page 48).
f)	PCR cycling conditions not optimal	Check the 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 F, page 48) 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. See Appendix A, page 37. 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.

		Comments and suggestions
i)	Primer design not optimal	Review primer design. Refer to Appendix B, page 38 for general guidelines on multiplex PCR primer design.
j)	Insufficient starting template	Increase the amount of starting template up to 300 ng per 25 µl reaction for gel-based detection and up to 200 ng per 25 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 C, page 40). 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 0.5 kb. Increase the extension time in increments of 30 s for each additional 0.5 kb of target sequence.
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 the protocols (see Tables 9 and 12 on pages 21 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 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 the functionality and specificity of primer pairs in singleplex 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 the QIAxcel or agarose gels, as this may affect multiplex PCR fidelity. Check the concentration of primer stock solutions (see Appendix C, page 40).
c)	PCR cycling conditions not optimal	Check that the correct cycling conditions were used (see Tables 9 and 12 on pages 21 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 F, page 48).

* 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.

		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 the protocols in (see Tables 9 and 12 on pages 21 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 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 9 and 12 on pages 21 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 F, page 48).
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 9 and 12 on pages 21 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 F, page 48).

		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 F, page 48).
d)	Mg^{2+} concentration not optimal	Use an initial Mg^{2+} concentration of 3 mM as provided by the Type-it 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 or agarose gels, as this may affect multiplex PCR fidelity. Check the concentration of primer stock solutions (see Appendix C, page 40).
f)	Primer design not optimal	Review primer design. Refer to Appendix B, page 38 for general guidelines on multiplex PCR primer design.

		Comments and suggestions
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 F, page 48).
h)	Primers degraded or of low quality	Check the functionality and specificity of primer pairs in singleplex 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 B, page 38 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 nondenaturing 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.

* 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.

		Comments and suggestions
b)	Too much starting template	Check the concentration of the starting template DNA (see Appendix C, Table 16, page 43). Repeat the multiplex PCR using less DNA (i.e., <300 ng per 25 µl reaction).
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 and 12 on pages 21 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 the heights <10000 relative fluorescent units on ABI PRISM 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.

		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 over water.
d)	n–1 products detected	Ensure that the final extension step was performed as described in Tables 9 and 12 on pages 21 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 the number of cycles by an increment of 1–2 cycles until all products are in the range of signals specified by the instrument manufacturer.
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.

	Comments and suggestions
	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: PCR Using Q-Solution

Both protocols include an option for using Q-Solution. Q-Solution changes the melting behavior of DNA and can be used for PCR systems that do not work well under standard conditions. When using Q-Solution for the first time for a particular primer–template pair, always perform parallel reactions with and without Q-Solution. This recommendation should also be followed if another PCR additive (such as DMSO) was previously used for a particular primer–template pair. It is not recommended to combine GC-rich target sequences that either amplify poorly or not at all with target sequences that amplify easily in a multiplex PCR. However, many primer–template systems can be combined in a multiplex PCR using this protocol.

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

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

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

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

Case D: Q-Solution causes reduced efficiency or failure of a previously successful amplification reaction. In this case, addition of Q-Solution disturbs the previously optimal primer–template annealing. Therefore, when using Q-Solution for the first time for a particular primer–template system, always perform reactions in parallel with and without Q-Solution.

Appendix B: 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 F, page 48). Otherwise, use the recommendations in Table 14.

Table 14. 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 the primer sequence is unique for your template sequence. Check similarity to other known sequences with BLAST® Software (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, (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 4, 5, and 6, pages 15 and 16), or by using primers labeled with different fluorescent dyes.

Appendix C: Handling and Storage of Primers and Template Quality

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.

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.

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

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 3, page 14).

Calculating the required dilution factor

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.

* 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.

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 3, page 14).

Template quality

Template quality is of extreme importance. Impurities have inhibitory effects on PCR. These are listed in Table 15.

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 15. 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 16 and 17, respectively. The Type-it Mutation Detect PCR Kit enables successful multiplex amplification using a wide range of template amounts down to the picogram level.

Table 16. 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 17. 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)	$2.7 \times 10_{9}$†	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 18. 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 D: Detection of Mutation Targets Amplified with the Type-it Mutation Detect PCR Kit Using Primers Labeled with Fluorescent Dyes

Fluorescent labels

Fluorescent labels should be chosen so that they are compatible with your detection instrument. We recommend choosing fluorescent labels according to the instuctions from the manufacturer of your detection instrument. See Appendix C, page 40 for general guidelines on handling and storage of fluorescently labeled primers.

Guidelines for analysis of mutation 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 the signal in the recommended signal intensity range as recommended by the instrument manufacturer.

For analysis of mutation targets amplified with the Type-it Mutation Detect PCR Kit using high-resolution sequencing instruments, such as the 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 19 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 19. 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)	Pure DNA
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 (Applied Biosystems) (Part Number: 4322682)	0.3 µl/reaction
	Add 0.2–0.5 µl/reaction	
Denaturation	Heat for 3–5 min at 95°C	5 min at 95°C
Time until loading	Load within 1 hour	Load within 1 hour

Appendix E: 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 Type-it Mutation Detect PCR Buffer increases specificity both at the start of and during PCR, making the Type-it Mutation Detect PCR 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, since 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 Type-it Mutation Detect PCR 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 F: Optimization of PCR Conditions for Special Multiplex PCR Applications

The Type-it Mutation Detect 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.

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 38, 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 C, page 40). The use of high-quality (e.g., HPLC purified) primers is recommended, although standard quality may also be sufficient. When using primers labeled with fluorescent dyes, 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 PCR products are not generated uniformly, check the concentration of all primers used in the multiplex assay (see Appendix C, page 40). 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 minutes instead of 90 seconds 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 47), doubling the concentration of this primer to 0.4 µM may lead to more uniform product yield.

Ordering Information

Product	Contents	Cat. no.
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
Type-it Mutation Detect PCR Kit (200)	For 200 x 25 µl reactions: Type-it Multiplex PCR Master Mix,* 5x Q-Solution, RNase-Free Water, and CoralLoad Dye	206343
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 Fast SNP Probe PCR Kit — for 5'-nuclease probe-based SNP detection with reliably high call rates		
Type-it Fast SNP Probe PCR Kit (100)†	For 100 x 25 µl reactions: 2x Type-it Fast SNP Probe PCR Master Mix,‡ 5x Q-Solution, RNase-Free Water	206042
Type-it HRM® PCR Kit — for accurate genotyping by High-Resolution Melting (HRM) analysis		
Type-it HRM PCR Kit (100)†	For 100 x 25 µl reactions: 1.3 ml of 2x HRM PCR Master Mix§ and RNase-Free Water	206542

* Contains HotStarTaq *Plus* DNA Polymerase, optimized $MgCl_2$ concentration, and 200 µM each dNTP.

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

‡ Contains HotStarTaq *Plus* DNA Polymerase, ROX dye, and dNTPs with optimized concentration of $MgCl_2$ and Q-Solution.

§ Contains HotStarTaq *Plus* DNA Polymerase, EvaGreen® dye, optimized concentration of Q-Solution, dNTPs, and $MgCl_2$.

Ordering Information

Product	Contents	Cat. no.
QIAGEN Multiplex PCR Kit — for fast and efficient multiplex PCR without optimization		
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
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
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

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

† Contains 15 mM $MgCl_2$.

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

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

Ordering Information

Product	Contents	Cat. no.
TopTaq® DNA Polymerase — for highly reliable end-point PCR with unrivaled ease-of-use		
TopTaq DNA Polymerase (250)*	250 units TopTaq DNA Polymerase, 10x PCR Buffer,† CoralLoad Concentrate, 5x Q-Solution, 25 mM $MgCl_2$	200203
TopTaq Master Mix Kit — for fast and convenient end-point PCR		
TopTaq Master Mix Kit (250)*	3 x 1.7 ml 2x TopTaq Master Mix‡ containing 250 units TopTaq DNA Polymerase in total, 1.2 ml 10x CoralLoad Concentrate, 3 x 1.9 ml RNase-Free Water; suitable for 200 x 50 µl reactions	200403
QIAGEN Fast Cycling PCR Kit — for rapid and highly specific PCR on any thermal cycler		
QIAGEN Fast Cycling PCR Kit (200)*	2 x 1 ml QIAGEN Fast Cycling PCR Master Mix, 10x CoralLoad Dye, Q-Solution, RNase-Free Water; suitable for 200 x 20 µl reactions.	203743
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

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

† Contains 15 mM $MgCl_2$.

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

Ordering Information

Product	Contents	Cat. no.
QIAamp DNA Kits — for purification of 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
MinElute® PCR Purification Kit — for purification of PCR products (70 bp to 4 kb) in low elution volumes		
MinElute PCR Purification Kit (50)*	50 MinElute Spin Columns, Buffers, Collection Tubes (2 ml)	28004
QIAquick® PCR Purification Kit — for purification of PCR products, 100 bp to 10 kb		
QIAquick PCR Purification Kit (50)*	50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28104
QIAquick Gel Extraction Kit — for gel extraction or cleanup of DNA (70 bp to 10 kb) from enzymatic reactions		
QIAquick Gel Extraction Kit (50)*	50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28704

Type-it Kits, HotStarTaq *Plus* Master Mix Kit, and HotStarTaq *Plus* DNA Polymerase are intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

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

Notes

Limited License Agreement

Use of this product signifies the agreement of any purchaser or user of the Type-it Mutation Detect PCR Kit to the following terms:

1. The Type-it Mutation Detect PCR Kit may be used solely in accordance with the *Type-it Mutation Detect PCR Handbook* and for use with components contained in the kit only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this kit with any components not included within this kit except as described in the *Type-it Mutation Detect PCR Handbook* and additional protocols available at www.qiagen.com.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this kit and/or its use(s) do not infringe the rights of third-parties.
3. This kit and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the kit agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the kit and/or its components.

For updated license terms, see www.qiagen.com.

www.qiagen.com

Australia ▪ Orders 03-9840-9800 ▪ Fax 03-9840-9888 ▪ Technical 1-800-243-066

Austria ▪ Orders 0800/28-10-10 ▪ Fax 0800/28-10-19 ▪ Technical 0800/28-10-11

Belgium ▪ Orders 0800-79612 ▪ Fax 0800-79611 ▪ Technical 0800-79556

Brazil ▪ Orders 0800-557779 ▪ Fax 55-11-5079-4001 ▪ Technical 0800-557779

Canada ▪ Orders 800-572-9613 ▪ Fax 800-713-5951 ▪ Technical 800-DNA-PREP (800-362-7737)

China ▪ Orders 021-3865-3865 ▪ Fax 021-3865-3965 ▪ Technical 800-988-0325

Denmark ▪ Orders 80-885945 ▪ Fax 80-885944 ▪ Technical 80-885942

Finland ▪ Orders 0800-914416 ▪ Fax 0800-914415 ▪ Technical 0800-914413

France ▪ Orders 01-60-920-926 ▪ Fax 01-60-920-925 ▪ Technical 01-60-920-930 ▪ Offers 01-60-920-928

Germany ▪ Orders 02103-29-12000 ° Fax 02103-29-22000 ° Technical 02103-29-12400

Hong Kong ▪ Orders 800 933 965 ▪ Fax 800 930 439 ▪ Technical 800 930 425

Ireland ▪ Orders 1800 555 049 ▪ Fax 1800 555 048 ▪ Technical 1800 555 061

Italy ▪ Orders 02-33430-420 ▪ Fax 02-33430-426 ▪ Technical 800-787980

Japan ▪ Telephone 03-6890-7300 ▪ Fax 03-5547-0818 ▪ Technical 03-6890-7300

Korea (South) ▪ Orders 1544 7145 ▪ Fax 1544 7146 ▪ Technical 1544 7145

Luxembourg ▪ Orders 8002-2076 ▪ Fax 8002-2073 ▪ Technical 8002-2067

Mexico ▪ Orders 01-800-7742-639 ▪ Fax 01-800-1122-330 ▪ Technical 01-800-7742-639

The Netherlands ▪ Orders 0800-0229592 ▪ Fax 0800-0229593 ▪ Technical 0800-0229602

Norway ▪ Orders 800-18859 ▪ Fax 800-18817 ▪ Technical 800-18712

Singapore ▪ Orders 65-67775366 ▪ Fax 65-67785177 ▪ Technical 65-67775366

Spain ▪ Orders 91-630-7050 ▪ Fax 91-630-5145 ▪ Technical 91-630-7050

Sweden ▪ Orders 020-790282 ▪ Fax 020-790582 ▪ Technical 020-798328

Switzerland ▪ Orders 055-254-22-11 ▪ Fax 055-254-22-13 ▪ Technical 055-254-22-12

UK ▪ Orders 01293-422-911 ▪ Fax 01293-422-922 ▪ Technical 01293-422-999

USA ▪ Orders 800-426-8157 ▪ Fax 800-718-2056 ▪ Technical 800-DNA-PREP (800-362-7737)

1060020 12/2009

Sample & Assay Technologies