December 2009

Type-it® Microsatellite PCR Handbook

For reliable multiplex PCR-based analysis of microsatellites without the need for optimization

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QIAGEN Sample and Assay Technologies

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Contents

Kit Contents

Type-it Microsatellite PCR Kit	**(70)**	**(200)**
Catalog no.	**206241**	**206243**
Number of 25 µl 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
Handbook	1	1

* Contains HotStarTaq® *Plus* DNA Polymerase, Type-it Microsatellite PCR Buffer with 6 mM $MgCl_2$, and dNTPs.

Shipping and Storage

The Type-it Microsatellite 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 Microsatellite 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 Microsatellite 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).

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 Microsatellite PCR Buffer:	Contains 6 mM $MgCl_2$; pH 8.7 (20°C).
dNTP Mix:	Contains dATP, dCTP, dGTP, dTTP; ultrapure quality
Q-Solution:	5x concentrated
RNase-free water:	Ultrapure quality, PCR-grade

Quality Control

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

Introduction

Accurate genotyping analysis often requires extensive optimization of experimental parameters. Sample materials may be limiting in genotyping studies, for example, 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) or other genetic information from an organism such as microsatellites. 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 SNPs to detection of mutations and identification of microsatellite loci.

The Type-it Microsatellite PCR Kit is specifically developed and functionally validated for multiplex PCR-based analysis of microsatellites or minisatellites such as STRs or VNTRs used, for example, in relationship analysis or population genetics (see Table 1, page 9).

This handbook contains protocols specially designed for detection of microsatellites with subsequent analysis on high-resolution capillary sequencers as well as on other detection platforms. The Type-it Microsatellite PCR Kit is available in an optimized, ready-to-use master mix format, simplifying multiplex PCR assay reaction setup.

Analysis of microsatellites

Eukaryotic genomes contain many repetitive elements. These repeated DNA sequences, often referred to as satellite DNA, exist in all sizes and differ both in the length and sequence of the repeat. The repeats are often highly polymorphic, with many different alleles present within a population. Alleles may also differ in the number of copies of a particular repeat sequence. Variation in satellite DNA sequences is important and can be used to determine genetic differences between organisms or the degree of identity between closely related individuals. Satellite loci are usually defined by the length of the core repeat unit and the number of repeats or the overall repeat length. The core repeat for a medium-length repeat is called a variable number tandem repeat (VNTR) or sometimes referred to as a minisatellite and is in the range of approximately 10–100 nucleotides in length.

DNA repeats of 2–6 nucleotides in length are called microsatellites, simple sequence repeats (SSRs), or short tandem repeats (STRs). The number of repeats in STR markers can be highly variable among individuals, making them a highly useful tool for individual identification, lineage analysis, or forensic investigations. Thousands of polymorphic microsatellites have been described in literature. STR or SSR markers occur throughout the whole genome every 10,000 nucleotides. A large number of STR, SSR, and VNTR markers have been characterized by both academic and commercial laboratories for use in prenatal diagnostics, population analysis, animal genotyping, or disease gene studies. Some STR markers are frequently used in human identity testing, paternity testing, and forensic investigations.

The Type-it Microsatellite PCR Kit has been developed as a generic tool for reliable and fast analysis of any STR or VNTR marker derived from human, animal, plants, and bacterial samples.

Fast and easy assay development

The Type-it Microsatellite PCR Kit is provided in a ready-to-use master mix format based on proven QIAGEN Multiplex Technology (patent pending), and 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 microsatellites and minisatellites such as STRs, SSRs, and VNTRs (see Table 1, page 9). 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 analysis of microsatellites on high-resolution capillary sequencers are included to ensure reliable results for routine analysis as well as for the establishment of new assays.

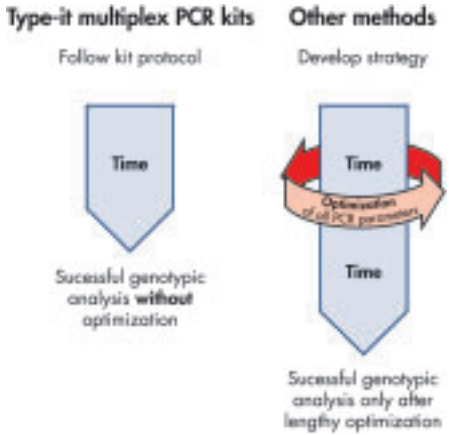

Figure 1. Successful genotypic analysis without the need for optimization. The Type-it Microsatellite PCR Kit is based on proven QIAGEN Multiplex Technology and provides a simple procedure for reliable genotyping results. In contrast to current methods, the kit eliminates the need for optimization of PCR parameters.

Table 1. Applications of the Type-it Microsatellite PCR Kit

Dedicated application	Field of research
Microsatellites	Typing of disease loci
STR (short tandem repeats)	Individual identification
SSR (simple sequence repeats)	Bacterial typing
VNTR (minisatellites)	Lineage analysis
	Population genetics

HotStarTaq *Plus* DNA Polymerase

The Type-it Multiplex PCR Master Mix contains HotStarTaq *Plus* DNA Polymerase, a chemically 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 hotstart enables reactions to be set up at room temperature, which is rapid and convenient.

Type-it Microsatellite PCR Buffer

The unique Type-it Microsatellite PCR Buffer facilitates the amplification of multiple PCR products. In contrast to conventional PCR reagents, the Type-it Microsatellite 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 Microsatellite 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 multiplex PCR-based microsatellite amplification and subsequent detection on capillary sequencers. It is nontoxic and PCR purity is guaranteed. For further information, read the protocol "Multiplex PCR for Amplification of Microsatellite Loci Using Q-Solution (Subsequent Analysis on Sequencing Instruments)", page 21.

Challenges in microsatellite analysis

Microsatellite analysis on high-resolution detection systems such as capillary sequencers is often associated with the problem of uneven product yield and huge intensity differences of fluorescent signals.

Optimization of the peak height on a capillary sequencer often leads to time-consuming variations of experimental conditions, often resulting in unstable assays and leading to insufficient genotyping results. The Type-it Microsatellite PCR Kit overcomes this problem by ensuring high product yields for all amplicons in a multiplex experiment.

Furthermore, during PCR of microsatellites, a number of challenges and artifacts can arise, such as stutter peaks, n–1 peaks, or allelic drop-out. These effects can interfere with clear interpretation of the genotyping results.

The Type-it Microsattelite PCR Kit is provided with optimized protocols for use with fluorescent primers, enabling subsequent analysis by high-resolution fragment analysis on capillary sequencers. This ensures reliable microsatellite analysis at the first attempt.

Stutter peaks

Amplification of microsatellite DNA may result in a low percentage of PCR products that are shorter than expected. Typically, the difference in size of these "stutter products" is exactly one repeat unit and these products are an intrinsic feature of the amplification of some satellite DNA sequences. For further recommendations on how to avoid stutter peaks, see Troubleshooting Guide, page 26.

n–1 products

Taq DNA polymerases, including HotStarTaq *Plus* DNA Polymerase, which is provided with the Type-it Multiplex PCR Master Mix, add an extra A residue to the 3' end of PCR products. When using the cycling protocol optimized for microsatellite analysis on high-resolution sequencer instruments (pages 17 and 21), all PCR products should have this additional A residue at the 3' end. However, if larger amounts of PCR product are generated using an increased number of cycles, it may be possible that some PCR products lack the extra A residue and n–1 products may be occur. For further recommendations on how to avoid n–1 peaks, see Troubleshooting Guide, page 26.

Allelic drop-out

If a base pair exchange occurs in the DNA primer-binding region due to a sequence polymorphism, the hybridization of the primer may be disrupted, resulting in failure to amplify and to detect an allele that was originally present in the template DNA.

The Type-it Microsatellite PCR Kit has been specifically optimized to minimize effects such as stutter peaks, n–1 products, and allelic drop-out, ensuring reliable and reproducible genotyping analysis.

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 Microsatellite 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 2). Primer quality is a crucial factor for successful multiplex PCR. Problems encountered in multiplex PCR are frequently due to the use of incorrect primer concentrations or low-quality primers.

Multiplex PCR of microsatellite loci is frequently performed using fluorescently labeled primers. Ensure that the fluorescent labels are compatible with the detection system used. Usually, microsatellites are detected using high-resolution sequencing instruments, such as the ABI PRISM® 3100, Applied Biosystems® 3130 or 3130xl, or Applied Biosystems 3730 or 3730xl Genetic Analyzer.

Different fluorescent dyes may give differing signal intensities on a particular detection instrument, although comparable amounts of PCR product are generated. We recommend combining fluorescent dyes for multiplex PCR according to the instructions of the detection instrument manufacturer. Primers labeled with fluorescent dyes should always be kept in the dark to prevent the fluorescent dye from bleaching. The use of HPLC-grade primers is recommended. Fluorescent labels should be chosen so that they are compatible with your detection instrument. See Appendix C and Appendix E, pages 39 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 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, 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

The detection of microsatellites amplified by the Type-it Microsatellite PCR Kit can be easily performed using high-resolution detection platforms such as sequencing instruments requiring fluorescently labeled primers, allowing resolution down to single bases.

Primer pairs for multiplex PCR analysis should be carefully designed. In addition to the sequence of the 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, PCR products can also be distinguished by their different dye labels, allowing analysis of fragments of the same size in the same reaction.

In addition to sequencing instruments, following amplification, microsatellites can be analyzed on capillary electrophoresis instruments such as the QIAxcel® System or the Agilent® 2100 Bioanalyzer, allowing resolution down to 3–5 bp.

Recommendations for the use of the Type-it Microsatellite PCR Kit with different detection systems are provided in Appendix A, page 35.

Guidelines for analysis of microsatellites on capillary sequencers

For successful analysis of multiplex PCR products derived with the Type-it Microsatellite 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
- ABI PRISM 377
- Beckman CEQ™ 8000 and CEQ 8800 Genetic Analysis Systems

For further details about analysis of multiplex PCR products on high-resolution sequencing instruments, see the protocols on pages 17 and 21.

Microsatellites are usually detected using capillary sequencer instruments. For recommendations on how to detect microsatellites on agarose gels or capillary electrophoresis instruments such as the QIAxcel, follow the recommendations in Appendix A, page 35.

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. REPLI-g® Kits, which amplify very small amounts of genomic DNA while introducing no sequence bias, can also be used. For more information about QIAamp, DNeasy, and 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 consideration for successful multiplex PCR of microsatellite loci.

For detailed information on template quality and quantity, see Appendix D, page 41.

Choosing the correct protocol

This handbook contains 2 protocols.

Multiplex PCR for Amplification of Microsatellite Loci (Subsequent Analysis on Sequencing Instruments).

Choose this protocol (page 17) for multiplex PCR for amplification of microsatellites that will be subsequently analyzed on capillary sequencer instruments.

Multiplex PCR for Amplification of Microsatellite Loci Using Q-Solution (Subsequent Analysis on Sequencing Instruments).

Choose this protocol (page 21) for multiplex PCR using Q-Solution for the amplification of microsatellites located in structural difficult regions (e.g., GC-rich) that will be subsequently analyzed on capillary sequencer instruments.

Q-Solution modifies the melting behaviour of DNA and can be used for PCR systems that do not work well under standard conditions. Choose this protocol for multiplex PCR for amplification of microsatellites that are difficult to amplify. This may be due to high GC content or a high degree of secondary structure.

Microsatellites are usually analyzed on capillary sequencer instruments. If you wish to analyze microsatellites using agarose-gel analysis, the QIAxcel System, or the Agilent 2100 Bioanalyzer system, refer to the recommendations in Appendix A, page 35.

Protocol: Multiplex PCR for Amplification of Microsatellite Loci (Subsequent Analysis on Sequencing Instruments)

Choose this protocol for multiplex PCR for amplification of microsatellites that will be subsequently analyzed on capillary sequencer instruments.

Important points before starting

- **Always start with the cycling conditions specified in this protocol.**
- **Note**: This protocol is designed for the detection of microsatellites on capillary sequencer instruments. If using other detection platforms, follow the recommendations in Appendix A, page 35.
- If using an already established microsatellite multiplex PCR assay system, use the previously established annealing temperature in combination with the cycling conditions specified in this protocol.
- **Annealing time must be 90 s.**
- 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.
- **Use equal concentrations (0.2 µM) of all primers.**
- **Prepare a 10x primer mix as described in Table 2, page 14.**
- 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, 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 3.**

   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 3.

   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 3. Reaction components for multiplex PCR of microsatellites

Component	Volume/reaction	Final concentration
Reaction mix		
2x Type-it Multiplex PCR Master Mix*	12.5 µl	1x
10x primer mix, 2 µM each primer (see Table 2)	2.5 µl	0.2 µM†
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	≤200 ng DNA Start with 10 ng
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 5 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 4.**

   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 4. Optimized cycling protocol for multiplex PCR amplification of microsatellites (for subsequent analysis on sequencers)*

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.‡
Number of cycles	28		Start with 28 cycles, which gives 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 5 for further recommendations.
Final extension	30 min	60°C	

* This protocol is optimized for subsequent analysis on sequencers. If using other detection systems, choose the cycling protocol in Table 13, page 37.

† 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 5. Recommendations for template amount and cycle number when analyzing amplicons using sequencers

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

7. **Analyze the samples using a capillary sequencer instrument.**

   Note: Prepare a dilution of 1:10 to 1:50 of your PCR product (1:10 is sufficient in most cases) before injecting it into a capillary sequencer. Dilute the PCR product using deionized formamide or water. Add up to 1 µl of undiluted sample per reaction.

   Note: A fluorescently labeled size standard must also be added to each sample before analysis. Any appropriate commercially available fluorescently labeled size standard can be used. Follow the supplier's instructions for the amount and handling of the size standard. Samples and size standard are less stable in water than in deionized formamide.

8. **Perform a denaturation step of 5 min at 95°C before injection into the sequencer.**

Protocol: Multiplex PCR for Amplification of Microsatellite Loci Using Q-Solution (Subsequent Analysis on Sequencing Instruments)

This protocol is designed for use with 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 with 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 with a particular primer–template pair.

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.

Important points before starting

- **Always start with the cycling conditions specified in this protocol.**
- This protocol is designed for the detection of microsatellites on capillary sequencer instruments. If using other detection platforms, follow the recommendations in Appendix A, page 35.
- **When using Q-Solution for the first time in a microsatellite PCR assay, it is important to perform parallel amplification reactions with and without Q-Solution (final concentration 0.5x).**
- If using an already established microsatellite multiplex PCR assay 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 B, page 38, for multiplex PCR primer design.
- **Prepare a 10x primer mix as described in Table 2, page 14.**
- 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, 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 6.**

   Note: The reaction mix typically contains all the components required for multiplex PCR of microsatellite loci 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 6.

   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 6. Reaction components for multiplex PCR of microsatellites using 2x Type-it Multiplex PCR Master Mix and Q-Solution

Component	Volume/reaction	Final concentration
Reaction mix		
2x Type-it Multiplex PCR Master Mix*	12.5 µl	1x
10x primer mix, 2 µM each primer (see Table 2)	2.5 µl	0.2 µM†
Optional: Q-Solution, 5x	2.5 µl	0.5x
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	≤200 ng DNA Start with 10 ng
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 8 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 7.**

   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 7. Optimized cycling protocol for multiplex PCR amplification of microsatellites using Q-Solution (for subsequent analysis on sequencers)*

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.‡
Number of cycles	28		Start with 28 cycles, which gives 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	30 min	60°C	

* This protocol is optimized for subsequent analysis on sequencers. If using other detection systems, choose the cycling protocol in Table 13, page 37.

† 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 8. Recommendations for template amount and cycle number if analyzing amplicons using sequencers

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

7. **Analyze the samples using a capillary sequencer instrument.**

   Note: Prepare a dilution of 1:10 to 1:50 of your PCR product (1:10 is sufficient in most cases) before injecting it into a capillary sequencer. Dilute the PCR product using deionized formamide or water. Add up to 1 µl of undiluted sample per reaction.

   Note: A fluorescently labeled size standard must also be added to each sample before analysis. Any appropriate commercially available fluorescently labeled size standard can be used. Follow the supplier's instructions for the amount and handling of the size standard. Samples and size standard are less stable in water than in deionized formamide.

8. **Perform a denaturation step of 5 min at 95°C before injection into the sequencer.**

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 18 and 23).
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 (≥10), 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. For calculation of the primer concentration, refer to Table 2, page 14.
d)	Insufficient number of cycles	Increase the number of PCR cycles. Refer to Table 5 and Table 8, pages 19 and 24, respectively.
e)	PCR cycling conditions not optimal	Check that the correct cycling conditions were used (see Tables 4 and 7 on pages 19 and 24, 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 44).

		Comments and suggestions
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 necessary, perform a gradient PCR (see Appendix F, page 44) to determine the optimal annealing temperature.
h)	GC-rich template or template with a high degree of secondary structure	Using the same cycling conditions, repeat the multiplex PCR using 0.5x Q-Solution. Follow the protocol on page 21. 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 B, page 38, for general guidelines on multiplex PCR primer design.
j)	Insufficient starting template	Increase the amount of starting template up to 200 ng per 25 µl reaction for sequencer-based detection and up to 300 ng per 25 µl reaction for agarose gel-based or QIAxcel detection.
k)	Low-quality starting template	Use only high-quality DNA, such as that purified using DNeasy Kits.

* 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
l)	Problems with starting template	Check the concentration, storage conditions, and quality of the starting template (see Appendix D, page 41). If necessary, make new serial dilutions of the 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. We recommend using an extension time of 2 min for target sequences of 1.5–2 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)	No final extension step, or final extension step was not optimal	Ensure that the final extension step was performed as described in Tables 4 and 7 on pages 19 and 24, 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.
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 the primer mix from primer stock solutions and store at –20°C in small aliquots. Avoid repeated freeze–thaw cycles of the primer mix.

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

		Comments and suggestions
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 39).
c)	PCR cycling conditions not optimal	Check that the correct cycling conditions were used (see Tables 4 and 7 on pages 19 and 24, 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 44).
d)	No final extension step, or final extension step was not optimal	Ensure that the final extension step was performed as described in Tables 4 and 7 on pages 19 and 24, 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 4 and 7 on pages 19 and 24, 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 44).
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. See the protocol on page 21.

		Comments and suggestions
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 4 and 7 on pages 19 and 24, 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 44).
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 44).
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.

		Comments and suggestions
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 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. For calculation of the primer concentration, refer to Appendix C, page 39.
f)	Primer design not optimal	Review primer design. Refer to Appendix B, page 38, 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.
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.

* 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	
Optimization of PCR conditions for analysis on capillary 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 (example: typical peak heights <10,000 relative fluorescent units on the ABI PRISM 3730 or 3730xl DNA Analyzer).
b)	Faint peaks ("stutter peaks")	Amplification of some microsatellite DNA sequences may lead to artifacts, referred to as stutter peaks, which are usually one repeat unit shorter than the main peak. We recommend decreasing the 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.
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 4 and 7 on pages 19 and 24, 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 analyzed. We recommend combining fluorescent dyes for multiplex PCR according to the instructions of the detection instrument's manufacturer.

	Comments and suggestions
Some products are missing in a multiplex experiment	
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.
Uneven product yield when analyzing multiplex PCR products on sequencing instruments	
a) Amount of template loaded is too high	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.
b) Uneven amplification of different products	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. Another approach is to increase the concentration of the weak primers to 1 µM final instead of 0.2 µM.
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.
Three or more peaks for one microsatellite marker	
DNA is contaminated or a mixture of DNAs has been analyzed	Check your DNA for impurities or contamination.

		Comments and suggestions
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.
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.
b)	Too much starting template	Check the concentration of the starting template DNA (see Table 16, page 42). Repeat the multiplex PCR using less DNA (i.e., <200ng 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 4 and 7 on pages 19 and 24, 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.

* 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.

Appendix A: Analysis of Microsatellites using Detection Platforms other than Capillary Sequencer Instruments

Table 9. Guidelines for analysis of multiplex PCR products obtained using the Type-it Microsatellite PCR Kit on different detection platforms

Detection platform	Number of cycles	Amount of template in the PCR reaction
Agilent 2100 Bioanalyzer	30–45	1–300 ng
Start with	35	100 ng
QIAxcel	30–45	1–300 ng
Start with	35	100 ng
Agarose gel	30–45	1–300 ng
Start with	35	100 ng

Agarose gel analysis

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

Table 10. Guidelines for agarose gel analysis of multiplex PCR products obtained using the Type-it Microsatellite PCR Kit

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%
20–50 bp	500 bp	2.5–3%
<20 bp†	250 bp	3.0–4%

* 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.

† 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 11. Guidelines for analysis of multiplex PCR products on 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

* 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.

Table 12. Guidelines for Agilent 2100 Bioanalyzer analysis of amplicons obtained using the Type-it Microsatellite PCR Kit

DNA LabChip® Kit	Sizing range	Sizing resolution	Sizing accuracy
1000	25–1000 bp†	5% from 100–500 bp 10% from 500–1000 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.

Table 13. Optimized cycling protocol for multiplex PCR amplification of microsatellites — subsequent analysis on agarose gels or the QIAxcel System or the Agilent 2100 Bioanalyzer*

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 starting the 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.
Final extension	10 min	68°C	

* This protocol is optimized for subsequent analysis on detection systems such as agarose gels or the QIAxcel System or the Agilent 2100 Bioanalyzer. If using capillary sequencers, choose the cycling protocols in Tables 4 and 7 on pages 19 and 24, respectively.

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

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 possible, perform a gradient PCR to determine the optimal annealing temperature (see page 44). 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 10, 11, and 12) or by using primers labeled with different fluorescent dyes.

Appendix C: 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 in 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.

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 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.

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

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 14).

Appendix D: Template Quality and Quantity

Quality of starting template

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 highly recommended for accurate PCR results.

* 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.

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 to 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 Microsatellite 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)	2.7×10^{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
$>1 \times 10^{3} - 5 \times 10^{4}$	1.1–55 fg	5.56–278 pg	3.6–179 ng
$>5 \times 10^{4}$	>55 fg	>278 pg	>179 ng

‡ Refers to single-copy genes.

Appendix E: Fluorescently Labeled Primers

Fluorescent labels should be chosen so that they are compatible with your detection instrument. We recommend choosing fluorescent labels according to the instructions of the manufacturer of your detection instrument. See Appendix C, page 39, for general guidelines on handling and storage of fluorescently labeled primers.

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

The Type-it Microsatellite 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 microsatellite loci amplification reactions with more than 10 different PCR products, an increase of the annealing time from 90 seconds to 3 minutes can improve product yield for primer pairs generating weak signals. 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 39). The use of high-quality (e.g., HPLC purified) fluorescently labeled primers is recommended.

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 C, page 39). 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.

Ordering Information

Product	Contents	Cat. no.
Type-it Microsatellite PCR Kit (70)	For 70 x 25 µl reactions: 2x Type-it Multiplex PCR Master Mix,* 5x Q-Solution, and RNase-Free Water	206241
Type-it Microsatellite PCR Kit (200)	For 200 x 25 µl reactions: 2x Type-it Multiplex PCR Master Mix,* 5x Q-Solution, and RNase-Free Water	206243
Related products		
Type-it Mutation Detect PCR Kit — for reliable detection of mutations by multiplex PCR		
Type-it Mutation Detect PCR Kit (70)†	For 70 x 25 µl reactions: 2x Type-it Multiplex PCR Master Mix,* 5x Q-Solution, RNase-Free Water, and CoralLoad® Dye	206341
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 the need for 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 (2 ml), RNase-Free Water (2 x 1.7 ml)	206143
HotStarTaq *Plus* DNA Polymerase — for highly specific hot-start PCR without the need for optimization		
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 Dye, 2 x 1.9 ml RNase-Free Water for 250 x 20 µl reactions	203643
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 Dye, 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 Dye, 3 x 1.9 ml RNase-Free Water; suitable for 200 x 50 µl reactions	200403

* 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.
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 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
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

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

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

Ordering Information

Product	Contents	Cat. no.
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
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

Notes

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