July 2009

Type-it® HRM™ PCR Handbook

For detection of gene mutations and SNPs by high-resolution melting (HRM) analysis

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

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Contents

Kit Contents

Type-it HRM PCR Kit	**(100)**	**(400)**
Catalog no.	**206542**	**206544**
Number of 25 µl reactions	**100**	**400**
2x HRM PCR Master Mix containing: ■ HotStarTaq® *Plus* DNA Polymerase ■ Type-it HRM PCR Buffer (with EvaGreen® dye) ■ Q-Solution® ■ dNTP mix (dATP, dCTP, dGTP, dTTP)	1 x 1.3 ml	4 x 1.3 ml
RNase-Free Water	1 x 2 ml	2 x 2 ml
Handbook	1	1

Storage

The Type-it HRM PCR Kit is shipped on dry ice. The kit should be stored immediately upon receipt at –20°C in a constant-temperature freezer and protected from light. When stored under these conditions and handled correctly, this product can be stored at least until the expiration date (see the inside of the kit lid) without showing any reduction in performance.

2x HRM 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 HRM PCR Kit is intended for molecular biology applications. This product is neither intended for the diagnosis, prevention, or treatment of a disease, nor has it been validated for such use either alone or in combination with other products. Therefore, the performance characteristics of the products for clinical use (i.e., diagnostic, prognostic, therapeutic, or blood banking) are unknown.

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 HRM 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 HRM 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.
Type-it HRM PCR Buffer:	Novel PCR buffer for highly specific amplification with subsequent high-resolution melting analysis.
EvaGreen:	Novel dsDNA-binding fluorescent dye, allowing highly efficient and inhibition-free PCR amplification and ideally suited for HRM analysis.
Q-Solution:	For successful amplification of difficult genomic loci. Contained in the master mix at optimized concentration.
dNTP mix:	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality.
RNase-free water:	Ultrapure quality, PCR-grade.

Quality Control

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

Introduction

The Type-it HRM PCR Kit is provided in a convenient master mix format for detection of gene mutations or SNPs via high-resolution melting analysis (HRM). HRM technology enables rapid characterization of DNA samples based on their melting behavior following PCR amplification. The kit contains the novel double-stranded DNA-binding fluorescent dye, EvaGreen, and includes an optimized HRM buffer and HotStarTaq *Plus* DNA Polymerase to eliminate nonspecific amplification products and provide reliable results. Q-Solution included in the master mix ensures specific amplification of difficult genomic loci, leading to successful results.

The Type-it HRM PCR Kit facilitates multiple applications, including:

- SNP genotyping
- Mutation discovery
- Scanning for mutations in disease and cancer-related genes
- Identification of candidate predisposition genes
- Genetic association studies
- DNA fingerprinting
- Species identification and genotyping

For DNA methylation analysis by HRM, use the EpiTect® HRM PCR Kit.

HRM (High-Resolution Melting)

High-resolution melting analysis is an innovative technique that is based on analysis of DNA melting. HRM characterizes DNA samples according to their dissociation behavior as they transition from double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA) with increasing temperature.

Before performing HRM analysis, the target sequence must be amplified to a high-copy number in the presence of the dsDNA-binding fluorescent dye, EvaGreen. The dye does not interact with ssDNA but actively binds to dsDNA and fluoresces brightly when bound. Change in fluorescence can be used to measure the increase in DNA concentration during PCR and then to directly measure thermally-induced DNA melting by HRM.

To perform high-resolution melting analysis, the temperature is increased from a lower to a higher temperature. The fluorescence of EvaGreen is measured continuously as the temperature is increased and is plotted against the temperature. EvaGreen fluoresces as long as it is bound to dsDNA. Due to the amplification procedure before the HRM analysis, fluorescence will be high at the beginning of the HRM analysis. Upon melting of dsDNA, EvaGreen is released and the fluorescence is reduced to a background level.

HRM is easier and more cost-effective than probe-based genotyping assays and, unlike conventional methods, it is a closed-tube system that prevents contamination with PCR products.

2x HRM PCR Master Mix

2x HRM PCR Master Mix ensures highly specific amplification, as well as flexible, rapid, and sensitive analysis of gene mutations and SNPs, and enables genotyping via high-resolution melting.

The components of 2x HRM PCR Master Mix include HotStarTaq *Plus* DNA Polymerase, Type-it HRM PCR Buffer, Q-Solution, and dNTPs. The optimized master mix ensures that the PCR products are amplified with high specificity and efficiency for successful HRM analysis even with difficult genomic loci.

HotStarTaq *Plus* DNA Polymerase

HotStarTaq *Plus* DNA Polymerase is a modified form of QIAGEN *Taq* DNA Polymerase, and is provided in an inactive state and has no enzymatic activity at ambient temperature. This prevents the formation of misprimed products and primer–dimers during reaction setup and the first denaturation step. Competition for reactants by PCR artifacts is therefore avoided, enabling high PCR specificity and accurate quantification. The enzyme is activated at the start of a reaction by a 5-minute, 95°C incubation step. The hot start enables reactions to be set up rapidly and conveniently at room temperature.

EvaGreen

EvaGreen is a novel fluorescent dye which selectively binds to dsDNA. Upon binding, fluorescence is strongly increased. The spectral properties of EvaGreen are very similar to those of SYBR® Green I. The absorbance maximum is 500 nm (with DNA bound) and the emission maximum is 530 nm. This allows easy detection of EvaGreen on channels/filters preset for HRM analysis and SYBR Green detection. In contrast to SYBR Green I, EvaGreen can be used in higher concentrations and shows equal binding affinity for GC-rich and AT-rich regions with no apparent sequence preference. This makes EvaGreen an ideal dye for HRM analyses of all types of PCR products.

Protocol selection

The Type-it HRM PCR Kit has been optimized for use with the following real-time cyclers: Rotor-Gene® Q, Rotor-Gene 6000, and LightCycler® 480.

This handbook contains 2 protocols for use with these cyclers. The Applied Biosystems® 7500 Fast System and Applied Biosystems 7900 can also be used with both optimized protocols in this handbook. Note that both instruments essentially require a dedicated calibration for the HRM dye in use before the experiment. A detailed protocol is provided at www.qiagen.com/Products/Type-itHRMPCRKit.aspx.

- "Protocol: Analysis of Gene Mutations and Microbial Genetic Differences" is found on page 14 and is recommended for all HRM applications except SNP genotyping.
- "Protocol: Analysis of SNPs by HRM" is found on page 21 and is recommended for SNP genotyping by HRM.

General considerations for genotyping by HRM

- Always check the real-time PCR instrument manual for details on HRM setup and analysis on your instrument.
- Note that conventional melting curve analysis (as done for SYBR Green-based detection) and HRM are not the same. HRM requires dedicated reaction chemistry as well as an HRM real-time PCR instrument with dedicated heating algorithm and software.

Template

- Purified genomic DNA of every origin suitable for PCR with respect to purity and concentration can be used to successfully perform HRM with the Type-it HRM PCR Kit.
- It is recommended to use the same genomic DNA purification procedure for all samples being analyzed by HRM. This avoids introduction of variations due to differing compositions of elution buffers used in different extraction methods.
- To avoid any reduction in performance, we recommend using QIAGEN genomic DNA purification kits such as QIAamp® or DNeasy® Kits.
- Use 1 ng to 50 ng of template genomic DNA or 1–50 pg microbial DNA per 25 µl reaction.
- **Important:** Use comparable amounts of template genomic DNA for all samples resulting in C_T values below 30 and differing by no more than 3 C_T values.
- It is recommended to use control DNA and sample DNA of comparable integrity. For example, for analyzing samples from FFPE-embedded tissues, control DNA should also be derived from FFPE tissues with comparable integrity.
- DNA samples used for HRM should be normalized in concentration. All DNA samples should be quantified and then adjusted to the same concentration using the same dilution buffer.

- Use sufficient PCR cycles so that all samples have reached the plateau phase of PCR to ensure that comparable amounts of PCR product are generated. Note that the amount of DNA affects the melting temperature of the PCR product. Check the protocols for details.

Assay design

Design assays with PCR product lengths of 70–350 bp. For SNP analysis, use of PCR products of 70–150 bp is recommended. Larger products can be analyzed successfully, but usually provide lower resolution. This is because, for example, a single base variation has a greater effect on the melting behavior of, for example, a 100 bp PCR product than on a 350 bp PCR product.

Primers

- Design primers allowing specific amplification. Perform a BLAST® search to ensure specific primer binding. See Appendix C, page 30 for details.
- The melting temperature of primers used for PCR with subsequent HRM analysis should be at least 56°C. 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.
- Check the concentration and integrity of primers before starting. Typically, standard primer quality primers are sufficient for HRM. For details, see Appendix C, page 30.
- **Important:** Always start with a primer concentration of 0.7 µM

HRM analysis

It is required to initially determine the melting point for each new HRM PCR product. Run HRM analysis to span a temperature range from 65°–95°C, covering the full range of expected melting points. In future experiments, after determination of the T_m, you may run HRM from 5°C below the lowest T_m of all expected PCR products to 5°C above the highest T_m of all PCR products in your experiment. This may reduce the time required for HRM analysis.

Data analysis

Check that the PCR result contains only specific product. Samples showing post-PCR artifacts such as primer–dimers or nonspecific products can make HRM results difficult to interpret. The Type-it HRM PCR Kit ensures maximum specificity with minimal need for optimization.

Cyclers

Rotor-Gene Q and Rotor-Gene 6000

QIAGEN's real-time PCR cycler, the Rotor-Gene Q, combines multiple optimized design features to provide outstanding performance and reliable results for demanding research applications.

The unique centrifugal rotary design of the Rotor-Gene Q makes it the most precise and versatile real-time PCR cycler currently available. Like the precursor model Rotor-Gene 6000, each tube spins in a chamber of moving air, keeping all samples at precisely the same temperature during rapid thermal cycling. Detection is similarly uniform. When each tube aligns with the detection optics, the sample is illuminated and the fluorescent signal is rapidly collected from a single, short optical pathway. This thermal and optical uniformity results in sensitive, precise, and fast HRM analysis. It also eliminates sample-to-sample variations and edge effects.

The comprehensive Rotor-Gene Q software package supports all current state-of-the-art HRM analysis procedures from basic to advanced algorithms.

Other real-time cyclers with HRM abilities

The LightCycler 480 as well as the Applied Biosystems 7500 Fast System and the Applied Biosystems 7900 System also enable HRM analysis with purchase of dedicated software. The cyclers are block cyclers and have the advantage of working with standard PCR plate formats. However, during HRM analysis, less reproducible and less precise high-resolution melt values are obtained due to temperature gradients across the block and multiple, complex optical pathways.

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.

- Primers: The Type-it HRM PCR Kit can be used with standard quality primers that can be purchased from established oligonucleotide manufacturers. Lyophilized primers should be dissolved in TE buffer to provide a stock solution of 100 µM; concentration should be checked by spectrophotometry. Primer solutions should be stored in aliquots at –20°C. Avoid repeated freeze/thaw cycles of primers. Prepare working solutions by dilution in TE (e.g., each primer at 10 µM) and store in small aliquots for single use.
- Nuclease-free (DNase-free) consumables: special care should be taken to avoid nuclease contamination of all reagents and consumables used to set up PCR.
- Optical PCR tubes or plates (use thin-walled PCR tubes or plates recommended by the manufacturer of your real-time cycler).
- **Optional:** Trizma® base and EDTA for preparing TE buffer for storing primers (see Appendix C, page 30). Use RNase/DNase-free water and plastic consumables to prepare TE buffer.

Important Notes

For optimal results with the Type-it HRM PCR Kit, the optimized protocols in the handbook **must be followed**. Following the protocols will ensure successful results without further optimization of PCR parameters such $MgCl_2$ concentration or annealing temperature.

No template control (NTC)

All detection experiments should include an NTC containing all the components of the reaction except for the template. This enables detection of potential contamination.

Positive control

Include at least one gDNA control of known genotype for each assay tested in the experiment to be used as reference. For a SNP genotyping experiment, at least one control is needed for each possible genotype (wild-type, heterozygote, variant).

Protocol: Analysis of Gene Mutations and Microbial Genetic Differences by HRM

This protocol is for use with the Rotor-Gene Q, Rotor-Gene 6000, and LightCycler 480. All protocols for HRM analysis for the specific instruments and upcoming HRM instruments are available online at www.qiagen.com/Products/Type-itHRMPCRKit.aspx.

Important points before starting

- The optimized protocols in the handbook **must be followed** to ensure successful results.
- **Always use a primer concentration of 0.7 µM.**
- No optimization of the Mg^{2+} concentration or the annealing temperature is required.
- **Always start with the cycling conditions specified in this protocol.**
- Optimal instrument and HRM analysis settings are a prerequisite for accurate genotyping results. For details, please refer to the manual provided with your HRM real-time PCR instrument.

Procedure

1. **Thaw 2x HRM PCR Master Mix, primer solutions, RNase-free water, template DNAs, and control DNAs (optional).**

   It is important to mix the solutions completely before use to avoid localized concentrations of salt.

2. **Prepare a reaction mix according to Table 1 (page 15).**

   It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed.

   Reaction setup can be done at room temperature (15–25°C). However, it is recommended to keep the individual reagents, samples, and controls on ice.

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or the wells of a PCR plate.**

4. **Add equal amounts and volumes of template DNA (1–50 ng genomic DNA or 1–50 pg microbial DNA, same amount for each sample) to the individual PCR tubes or wells and mix thoroughly.**

   Add sufficient DNA so that all samples show C_T values below 30. Samples should not differ by more than three C_T values.

Table 1. Reaction composition using 2x HRM PCR Master Mix

Component	Volume per 25 µl reaction*	Volume per 10 µl reaction (384-well plate)	Final concentration
Reaction mix			
2x HRM PCR Master Mix	12.5 µl	5.0 µl	1x
10 µM primer mix†	1.75 µl	0.7 µl	0.7 µM forward primer 0.7 µM reverse primer
RNase-free water	Variable	Variable	–
Template DNA (added at step 4)	Variable (equal volume for all reactions)	Variable (equal volume for all reactions)	Eukaryotic: 1–50 ng DNA/reaction Microbial: 1–50 pg DNA/reaction (use equal amounts for each reaction)
Total volume per reaction	**25 µl***	**10 µl**	**–**

* If your real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly.

† A 10 µM primer mix consists of 10 µM forward primer and 10 µM reverse primer.

5. **Program the real-time cycler according to Table 2 or 3 (pages 16–18).**

   Note: Check the real-time cycler's user manual for correct instrument setup.

6. **Place the PCR tubes or plate in the real-time cycler, and start the PCR cycling program, followed by HRM analysis.**

7. **Perform data analysis.**

   Before performing data analysis for real-time PCR and HRM, specify the analysis settings. See the real-time PCR instrument manual for details.

   Note: Real-time cyclers not listed in this handbook often do not have the ability to perform HRM analysis.

Table 2. Optimized cycling protocol for HRM analysis on the Rotor-Gene Q and Rotor-Gene 6000

Step	Time	Temp.	Additional comments
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
3-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**10 s**	**95°C**	
Annealing	**30 s**	**55°C**	
Extension	**10 s**	**72°C**	Activate fluorescence data acquisition on the green channel. Suitable for PCR products up to 350 bp. For PCR products >350 bp, use 1 s extension time per 25 bp of PCR product length.
Number of cycles	40		10–50 ng template DNA or 10–50 pg microbial DNA
	45		1–9 ng template DNA or 1–9 pg microbial DNA
HRM	2 s	65–95°C* 0.1°C increments	Fluorescence data acquisition; for details, see page 10.

* It is required to initially determine the melting point for each new HRM PCR product. Run HRM analysis to span a temperature range from 65°C–95°C, covering the full range of expected melting points. In future experiments, after determination of the T_m, you may run HRM from 5°C below the lowest T_m of all expected PCR products to 5°C above the highest T_m of all PCR products in your experiment. This may reduce the time required for HRM analysis.

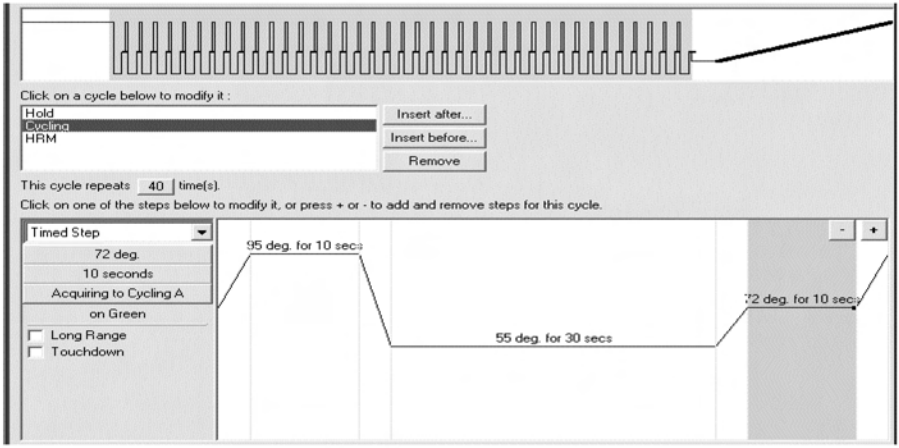

Figure 1. Programming the cycling protocol on the Rotor-Gene system for gene mutation analysis. Important: Set the "hold" step to 5 min at 95°C to activate HotStarTaq *Plus* DNA Polymerase (not shown). For the cycling steps, use 95°C for 10 s, 55°C for 30 s, and 72°C for10 s, with data acquiring to Cycling A on the green channel for the 72°C step. Adjust the number of cycles; use 40 as a starting point. Refer to Table 2 for details. Use maximum heating/cooling rates for all steps. Programming of the HRM protocol is shown in Figure 2.

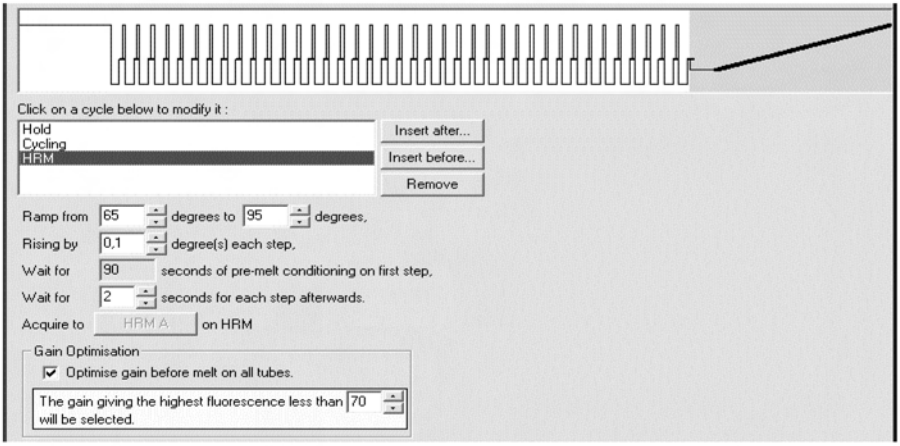

Figure 2. Programming the Rotor-Gene system for HRM analysis. Program the cycling protocol for gene mutation analysis described in Figure 1 and Table 2. For HRM analysis, ramp from 65°C to 95°C, rising by 0.1°C each step.

Table 3. Optimized cycling protocol for HRM analysis on the LightCycler 480

Step	Time	Temp.	Additional comments
			Important: Choose detection format: SYBR Green I/HRM Dye
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
3-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**10 s**	**95°C**	
Annealing	**30 s**	**55°C**	
Extension	**10 s**	**72°C**	Activate "single" fluorescence data acquisition. Suitable for PCR products up to 350 bp. For PCR products >350 bp, use 1 s extension time per 25 bp of PCR product length.
Number of cycles	45		10–50 ng template DNA or 10–50 pg microbial DNA
	50		1–9 ng template DNA or 1–9 pg microbial DNA
HRM			Analysis mode: Melting curve
	1 s	65°C*	
		95°C*	Continuous fluorescence data acquisition. Ramp rate: 0.02°C/s 25 acquisitions per second
Cooling	1 s	40°C	Cooling samples after HRM

Note: Use maximum ramp rates for heating and cooling.

* It is required to initially determine the melting point for each new HRM PCR product. Run HRM analysis to span a temperature range from 65°C–95°C, covering the full range of expected melting points. In future experiments, after determination of the T_m, you may run HRM from 5°C below the lowest T_m of all expected PCR products to 5°C above the highest T_m of all PCR products in your experiment. This may reduce the time required for HRM analysis.

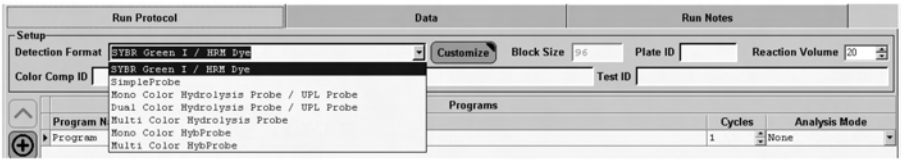

Figure 3. Programming the LightCycler 480 for HRM analysis. Choose detection format: SYBR Green I/HRM Dye. **Note:** If this channel for detection of EvaGreen dye is not chosen, reduced fluorescence may be observed.

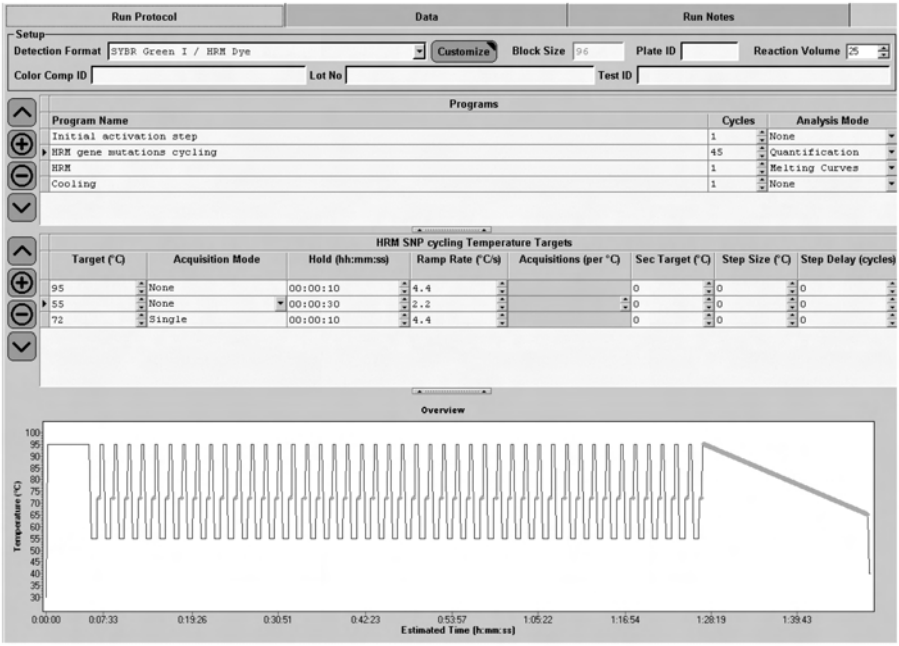

Figure 4. Programming the cycling protocol on the LightCycler 480 system for gene mutation analysis. Note: Times are entered in the format hh:mm:ss. **Important:** Set the initial activation step to 00:05:00 (5 min) at 95°C to activate HotStarTaq *Plus* DNA Polymerase (not shown). For the cycling steps, use 00:00:10 (10 s) at 95°C, 00:00:30 (30 s) at 55°C, and 00:00:10 (10s) at 72°C. Select "Single" for "Acquisition Mode" only for the 72°C step. Adjust the number of cycles; use 45 as a starting point. Refer to Table 3 for details. Use maximum heating/cooling rates for all steps. Programming of the HRM protocol is shown in Figure 5. Following HRM, samples should be cooled after the last cycle by entering 00:00:01 (1 s) at 40°C (not shown).

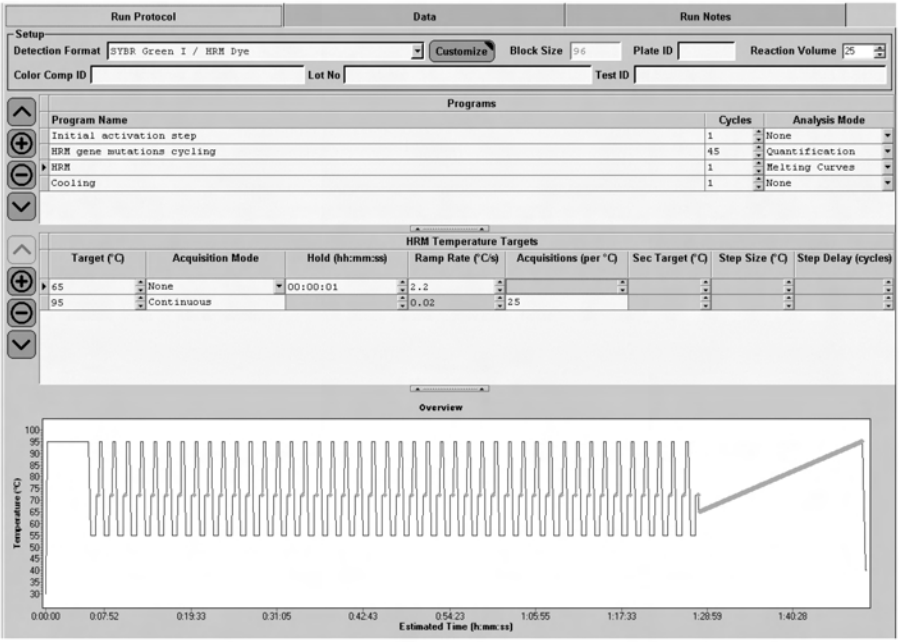

Figure 5. Programming the LightCycler 480 system for HRM analysis. Note: Times are entered in the format hh:mm:ss. Program the cycling protocol described in Figure 4. For HRM analysis, use 00:00:01 (1 s) at 65°C and 95°C. Select "Continuous" for "Acquisition Mode" with 25 acquisitions per second, resulting in a ramp rate of 0.02°C/s. Following HRM, samples should be cooled after the last cycle by entering 00:00:01 (1 s) at 40°C (not shown).

Protocol: Analysis of SNPs by HRM

This protocol is for use with the Rotor-Gene Q, Rotor-Gene 6000, and LightCycler 480. All protocols for HRM analysis for the specific instruments and upcoming HRM instruments are available online at www.qiagen.com/Products/Type-itHRMPCRKit.aspx.

Important points before starting

- The optimized protocols in the handbook **must be followed** to ensure successful results.
- **Always use a primer concentration of 0.7 µM.**
- No optimization of the Mg^{2+} concentration or the annealing temperature is required.
- Always start with the cycling conditions specified in this protocol.
- Optimal instrument and HRM analysis settings are a prerequisite for accurate genotyping results. For details, please refer to the manual provided with your HRM real-time PCR instrument.

Procedure

1. **Thaw 2x HRM PCR Master Mix, primer solutions, RNase-free water, template DNAs, and control DNAs (optional).**

   It is important to mix the solutions completely before use to avoid localized concentrations of salt.

2. **Prepare a reaction mix according to Table 4 (page 22).**

   It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed.

   Reaction setup can be done at room temperature (15–25°C). However, it is recommended to keep the individual reagents, samples, and controls on ice.

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or the wells of a PCR plate.**

4. **Add equal amounts and volumes of template DNA (1–50 ng genomic DNA or 1–50 pg microbial DNA, same amount for each sample) to the individual PCR tubes or wells and mix thoroughly.**

   Add sufficient DNA so that all samples show C_T values below 30. Samples should not differ by more than three C_T values.

Table 4. Reaction composition using 2x HRM PCR Master Mix

Component	Volume per 25 µl reaction*	Volume per 10 µl reaction (384-well plate)	Final concentration
Reaction mix			
2x HRM PCR Master Mix	12.5 µl	5.0 µl	1x
10 µM primer mix†	1.75 µl	0.7 µl	0.7 µM forward primer 0.7 µM reverse primer
RNase-free water	Variable	Variable	–
Template DNA (added at step 4)	Variable (equal volume for all reactions)	Variable (equal volume for all reactions)	Eukaryotic: 1–50 ng DNA/reaction Microbial: 1–50 pg DNA/reaction (use equal amounts for each reaction)
Total volume per reaction	**25 µl***	**10 µl**	**–**

* If your real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly.

† A 10 µM primer mix consists of 10 µM forward primer and 10 µM reverse primer.

5. **Program the real-time cycler according to Table 5 or 6 (pages 23– 25).**

   Note: Check the real-time cycler's user manual for correct instrument setup.

6. **Place the PCR tubes or plate in the real-time cycler, and start the PCR cycling program, followed by HRM analysis.**

7. **Perform data analysis.**

   Before performing data analysis for real-time PCR and HRM, specify the analysis settings. See the real-time PCR instrument manual for details.

   Note: Real-time cyclers not listed in this handbook often do not have the ability to perform HRM analysis.

Table 5. Optimized cycling protocol for HRM analysis of SNPs on the Rotor-Gene Q and Rotor-Gene 6000

Step	Time	Temp.	Additional comments
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**10 s**	**95°C**	
Annealing/Extension	**30 s**	**55°C**	Activate fluorescence data acquisition on the green channel. Suitable for PCR products up to 200 bp. For PCR products >200 bp, use additional 1 s extension time per 25 bp of PCR product length.
Number of cycles	40		10–50 ng template DNA or 10–50 pg microbial DNA
	45		1–9 ng template DNA or 1–9 pg microbial DNA
HRM	2 s	65–95°C* 0.1°C increments	Fluorescence data acquisition.

* It is required to initially determine the melting point for each new HRM PCR product. Run HRM analysis to span a temperature range from 65°C–95°C, covering the full range of expected melting points. In future experiments, after determination of the T_m, you may run HRM from 5°C below the lowest T_m of all expected PCR products to 5°C above the highest T_m of all PCR products in your experiment. This may reduce the time required for HRM analysis.

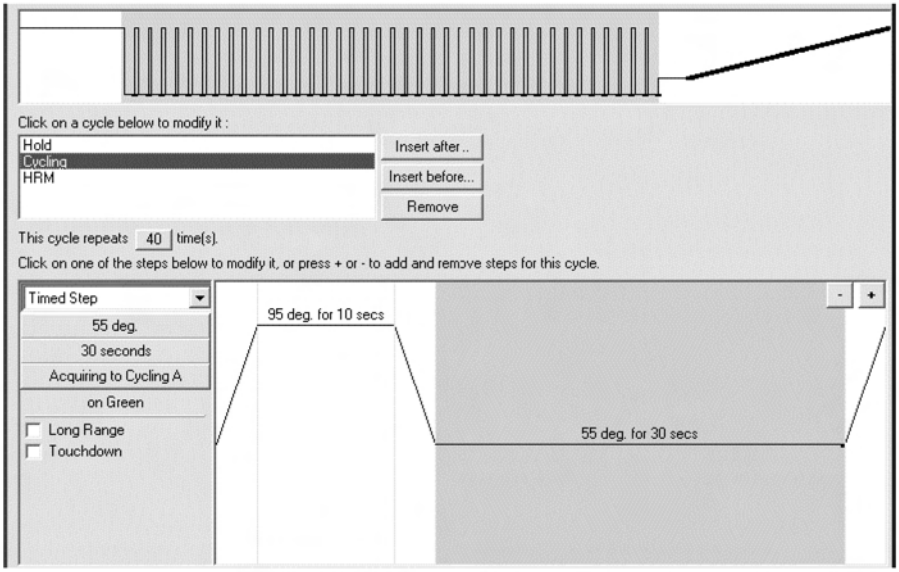

Figure 6. Programming the cycling protocol on the Rotor-Gene system for SNP analysis. Important: Set the "hold" step to 5 min at 95°C to activate HotStarTaq *Plus* DNA Polymerase (not shown). For the cycling steps, use 95°C for 10 s and 55°C for 30 s, with data acquiring to Cycling A on the green channel for the 55°C step. Adjust the number of cycles; use 40 as a starting point. Refer to Table 5 for details. Use maximum heating/cooling rates for all steps. Programming of the HRM protocol is shown in Figure 7.

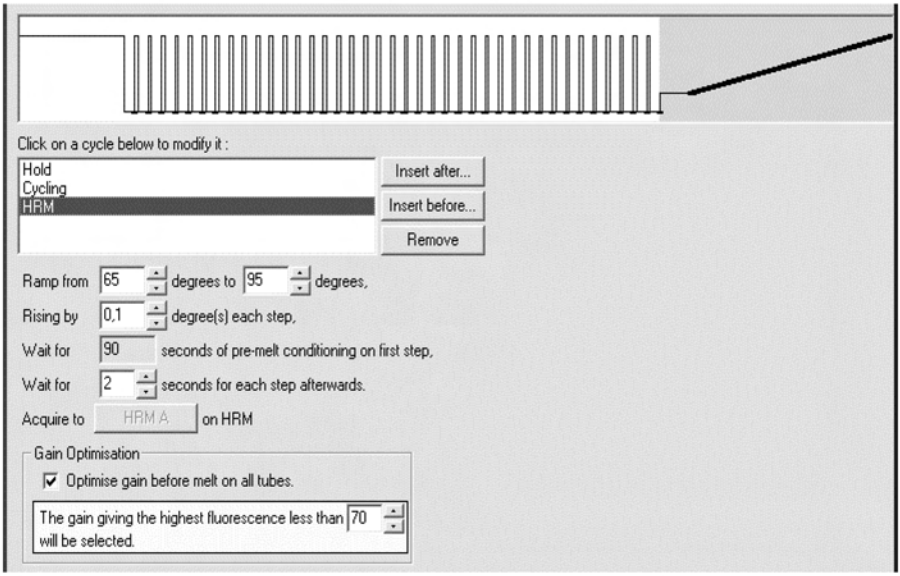

Figure 7. Programming the Rotor-Gene system for HRM analysis. Program the cycling protocol described in Figure 6 and Table 5. For HRM analysis, ramp from 65°C to 95°C, rising by 0.1°C each step.

Table 6. Optimized cycling protocol for HRM analysis of SNPs on the LightCycler 480

Step	Time	Temp.	Additional comments
			Important: Choose detection format: SYBR Green I/HRM Dye
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**10 s**	**95°C**	
Annealing/Extension	**30 s**	**55°C**	Activate "single" fluorescence data acquisition. Suitable for PCR products up to 200 bp. For PCR products >200 bp, use additional 1 s extension time per 25 bp of PCR product length.
Number of cycles	45		10–50 ng template DNA or 10–50 pg microbial DNA
	50		1–9 ng template DNA or 1–9 pg microbial DNA
HRM			Analysis mode: Melting curve
	1 s	65°C*	
		95°C*	Continuous fluorescence data acquisition. Ramp rate: 0.02°C/s 25 acquisitions per second
Cooling	1 s	40°C	Cooling samples after HRM

Note: Use maximum ramp rates for heating and cooling.

* It is required to initially determine the melting point for each new HRM PCR product. Run HRM analysis to span a temperature range from 65°C–95°C, covering the full range of expected melting points. In future experiments, after determination of the T_m, you may run HRM from 5°C below the lowest T_m of all expected PCR products to 5°C above the highest T_m of all PCR products in your experiment. This may reduce the time required for HRM analysis.

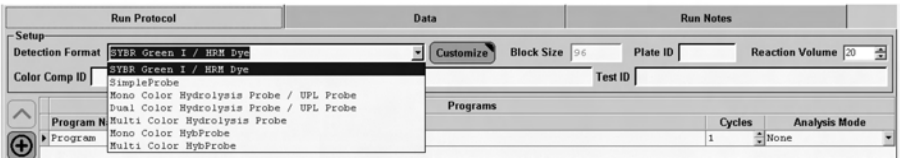

Figure 8. Programming the LightCycler 480 for HRM analysis. Choose detection format: SYBR Green I/HRM Dye. **Note:** If this channel for detection of EvaGreen dye is not chosen, reduced fluorescence may be observed.

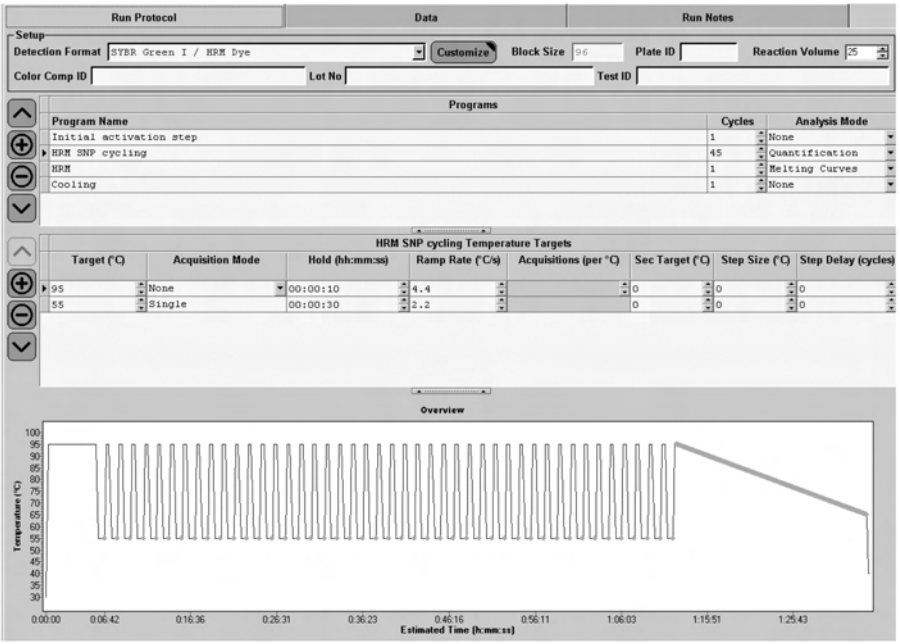

Figure 9. Programming the cycling protocol on the LightCycler 480 system for SNP analysis. Note: Times are entered in the format hh:mm:ss. **Important:** Set the initial activation step to 00:05:00 (5 min) at 95°C to activate HotStarTaq *Plus* DNA Polymerase (not shown). For the cycling steps, use 00:00:10 (10 s) at 95°C and 00:00:30 (30 s) at 55°C. Select "Single" for "Acquisition Mode" only for the 55°C step. Adjust the number of cycles; use 45 as a starting point. Refer to Table 6 for details. Use maximum heating/cooling rates for all steps. Programming of the HRM protocol is shown in Figure 10. Following HRM, samples should be cooled after the last cycle by entering 00:00:01 (1 s) at 40°C (not shown).

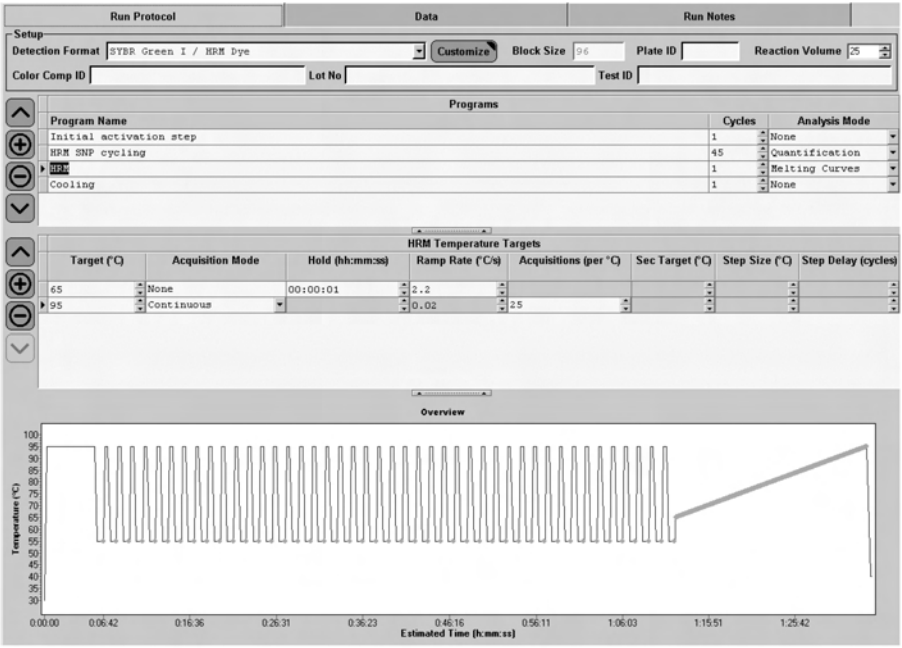

Figure 10. Programming the LightCycler 480 system for HRM analysis. Note: Times are entered in the format hh:mm:ss. Program the cycling protocol described in Figure 9. For HRM analysis, use 00:00:01 (1 s) at 65°C and 95°C. Select "Continuous" for "Acquisition Mode" with 25 acquisitions per second, resulting in a ramp rate of 0.02°C/s. Following HRM, samples should be cooled after the last cycle by entering 00:00:01 (1 s) at 40°C (not shown).

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
No signal, poor R_n value (PCR or HRM) or signal detected late in PCR		
a)	Wrong cycling conditions	Always start with the optimized cycling conditions specified in the protocols.
b)	HotStarTaq *Plus* DNA Polymerase not activated	Ensure that the cycling program includes the HotStarTaq *Plus* DNA Polymerase activation step (5 min at 95°C) as described in the protocols.
c)	Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers and template nucleic acid. See Appendix C (page 30) for details on evaluating the concentration of primers. Repeat the assay.
d)	Wrong or no detection step in real-time analysis	For real-time analysis, ensure that fluorescence detection takes place during the 72°C extension step for the gene mutation protocols or during the combined annealing/extension step for the SNP protocol.
e)	Poor PCR efficiency	Use the primer concentrations given in the protocol. See Appendix C (page 30) for details on determining the concentration of primers. Avoid repeated freezing and thawing of primers. Prepare small aliquots and only thaw a few times.
f)	Problems with starting template DNA	Check the concentration, storage conditions, and quality of the template and control DNA. If necessary, make new serial dilutions of the template DNA from the stock solutions. Repeat the assay using the new dilutions. Efficient removal of PCR inhibitors is essential for optimal results. Purify nucleic acids from your sample using an appropriate purification method. Ensure that all reagents, buffers and solutions used for isolating and dilution of template nucleic acids are free of nucleases (RNases/DNases).

		Comments and suggestions
g)	Insufficient or degraded template DNA	Check if template amount and PCR cycle number were used as specified in the protocol (Tables 1–6). Increase the amount of template DNA if possible.
Increased fluorescence or C_T value for "No Template" control		
a)	Contamination of reagents	Discard all the components of the HRM PCR assay (e.g., master mix, primers). Repeat the assay using new components and decontaminated pipettes and consumables.
b)	Contamination of real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions.
Variability in signal (C_T and/or R_n in HRM) between replicates or samples		
a)	Problem with template DNA	Recheck the DNA concentrations of the samples. Ensure that comparable amounts of DNA are used in all samples, noting that all samples should not differ by more than three C_T values. Use a different HRM genotyping assay to check the integrity of the genomic DNA in all samples. Note that rare or new genetic variants may generate results outside of the expected ranges.
b)	Bubbles in the wells	Spin down plates to remove air bubbles and remove any liquid from the plate cover before PCR.
c)	Reaction components improperly mixed	Follow mixing procedures in the protocol.
d)	Contamination of real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions
e)	Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.

Appendix A: HRM Instrument Setup and Data Analysis

Please refer to your real time cycler user manual for correct instrument setup and data analysis.

If using the Rotor-Gene Q, refer to section 11 (HRM data analysis) of the *Rotor-Gene Q User Manual*.

Note that in most cases the "difference plot" is required for accurate data analysis and genotyping results. Melting peaks alone will not provide accurate results in many cases.

Appendix B: Starting Template

Sample degradation should be avoided during purification and storage. Avoid excessive amounts of inhibitors from ethanol carryover. To improve HRM results, we recommend keeping the amount of template used consistent between samples. Spectrophotometric analysis for determining DNA concentration and purity is recommended. We recommend QIAGEN kits for sample preparation.

Note: At 260 nm, one absorbance unit is equal to 50 µg/ml DNA. Pure DNA will provide a 260 nm to 280 nm ratio of 1.8.

Appendix C: Designing and Handling Primers

Sequence

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

- Primers for HRM PCR should be 18–30 nucleotides in length.
- The melting temperature of primers used for HRM PCR should be at least 56°C. 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.
- Primers for HRM PCR should have a GC content of 40–60%.
- Avoid complementarity of 2 or 3 bases at the 3' ends of primer pairs to reduce primer–dimer formation.
- Avoid mismatches between the 3' end of the primer and the target-template sequence.
- Avoid runs of 3 or more G and/or C at the 3' end.
- Avoid complementary sequences within primers and between primer pairs.
- Ensure primer sequence is unique for your template sequence. Check similarity to other known sequences with BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) or primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/).

Commercially available computer software (e.g., OLIGO 6, Rychlik, 1999) or web-based tools such as Primer3, Steve Rosen & Helen Skaletsky, 2000, (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) can also be used for primer design.

Handling and storing primers

Guidelines for handling and storing primers are given below. For optimal results, we recommend only combining primers of comparable quality.

Storage buffer

Lyophilized primers should be dissolved in a small volume of low-salt buffer to give a concentrated stock solution (e.g., 100 µM). We recommend using TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) for standard primers.

Storage

Primers should be stored in sterile, nuclease-free TE buffer in small aliquots at –20°C. Standard primers are stable under these conditions for at least 1 year.

Dissolving primers

Before opening a tube containing lyophilized primer, centrifuge the tube briefly to collect all material at the bottom of the tube. To dissolve the primer, add the required volume of sterile, nuclease-free TE buffer, mix, and leave for 20 minutes to allow the primer to completely dissolve. Mix again and determine the concentration by spectrophotometry as described below.

We do not recommend dissolving primers in water. They are less stable in water than in TE buffer and some may not dissolve easily in water.

Concentration

Spectrophotometric conversion for primers:

1 A_{260} unit = 20–30 µg/ml

To check primer concentration, the molar extinction coefficient (ε_{260}) can be used:

$A_{260} = \varepsilon_{260}$ x molar concentration of primer or probe

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

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

Example

Concentration of diluted primer: 1 µM = 1 x 10^{-6} M

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

Calculation of expected A_{260}: 0.89 x [(6 x 15,480) + (6 x 7340) + (6 x 11,760) + (6 x 8850)] x (1 x 10^{-6}) = 0.232

The measured A_{260} should be within ±30% of the theoretical value. If the measured A_{260} is very different to the theoretical value, we recommend recalculating the concentration of the primers, or having the primers resynthesized.

Ordering Information

Product	Contents	Cat. no.
Type-it HRM PCR Kit (100)	For 100 x 25 µl reactions: 2x HRM PCR Master Mix (containing HotStarTaq *Plus* DNA Polymerase, EvaGreen dye, Q-Solution, dNTPs, and optimized concentration of $MgCl_2$) and RNase-free water	206542
Type-it HRM PCR Kit (400)	For 400 x 25 µl reactions: 2x HRM PCR Master Mix (containing HotStarTaq *Plus* DNA Polymerase, EvaGreen dye, Q-Solution, dNTPs, and optimized concentration of $MgCl_2$) and RNase-free water	206544
Related products		
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 Fast SNP Probe PCR Master Mix,† 5x Q-Solution, RNase-Free Water	206042
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: Multiplex PCR Master Mix,‡ 5x Q-Solution, RNase-Free Water, and 10x CoralLoad® Dye	206341
Type-it Microsatellite PCR Kit — for reliable microsatellite analysis by multiplex PCR		
Type-it Microsatellite PCR Kit (70)*	For 70 x 25 µl reactions: Multiplex PCR Master Mix,‡ 5x Q-Solution, and RNase-Free Water	206241

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

† Master mix contains HotStarTaq *Plus* DNA Polymerase, ROX dye, and dNTPs with optimized concentration of $MgCl_2$ and Q-Solution.

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

Ordering Information

Product	Contents	Cat. no.
HotStarTaq *Plus* DNA Polymerase — for highly specific hot-start PCR without 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 Concentrate, 2 x 1.9 ml RNase-Free Water for 250 x 20 µl reactions	203643
QIAamp DNA Kits — for genomic, mitochondrial, bacterial, parasite, or viral DNA		
QIAamp DNA Mini Kit (50)*	For 50 DNA preps: 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, Reagents, Buffers, Collection Tubes (2 ml)	51304
DNeasy Blood & Tissue Kits — for purification of total DNA from animal blood and tissues, and from cells, yeast, bacteria, or viruses		
DNeasy Blood & Tissue Kit (50)*	50 DNeasy Mini Spin Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	69504

The Type-it Fast SNP Probe PCR Kit, the Type-it Mutation Detect PCR Kit, and the Type-it Microsatellite Kit 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 available; see www.qiagen.com.

† Contains 15 mM $MgCl_2$.

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

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