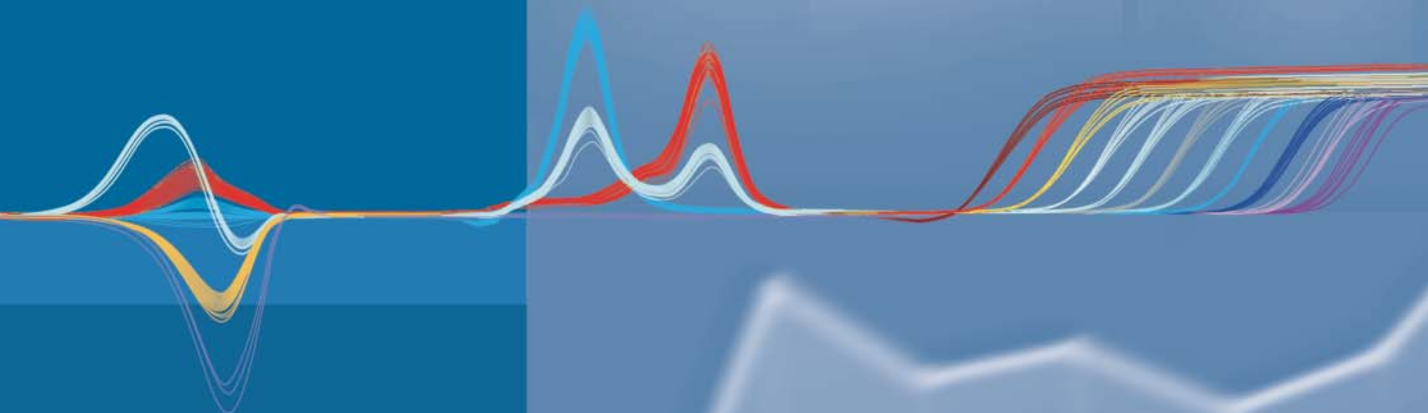
LightCycler® Real-Time PCR Systems

Application Manual



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Preface

This application manual serves as a general introduction into the principles of Real-Time PCR and as a guide to the highly diverse applications of this method in life science research. As reflected by the book´s structure, it covers both theoretical and practical aspects:

I. The first chapter provides an overview of PCR history, starting from the early years of discovery of the PCR principle and leading on to developments and modern methods for monitoring and analyzing reactions online and in real-time. It also introduces the assay formats most commonly used on today´s Real-Time PCR platforms.

II. The second chapter provides a more detailed introduction into Real-Time PCR applications, from gene detection and identification in virology and microbiology, discovery and detection of genetic variation, to - last but not least - absolute and relative quantification in gene expression analysis.

III. Since the success and scientific accuracy of results obtained from any PCR experiment will always depend on assay design and experimental planning, the third chapter provides advanced information on this topic. It should help users identify critical steps in their workflows and provide starting points for optimization, in case experimental results are not satisfactory.

IV. The forth chapter finally offers a collection of case studies provided by users of the LightCycler® 480 Real-Time PCR System, who describe the use of this high-throughput platform in their real-life, daily research work.

Roche Applied Science is delighted to offer biomedical researchers using Real-Time PCR its support on the level of technical and scientific information about this powerful and still developing technology, as well as our commitment to continuous research and development of new instruments, software and reagents.

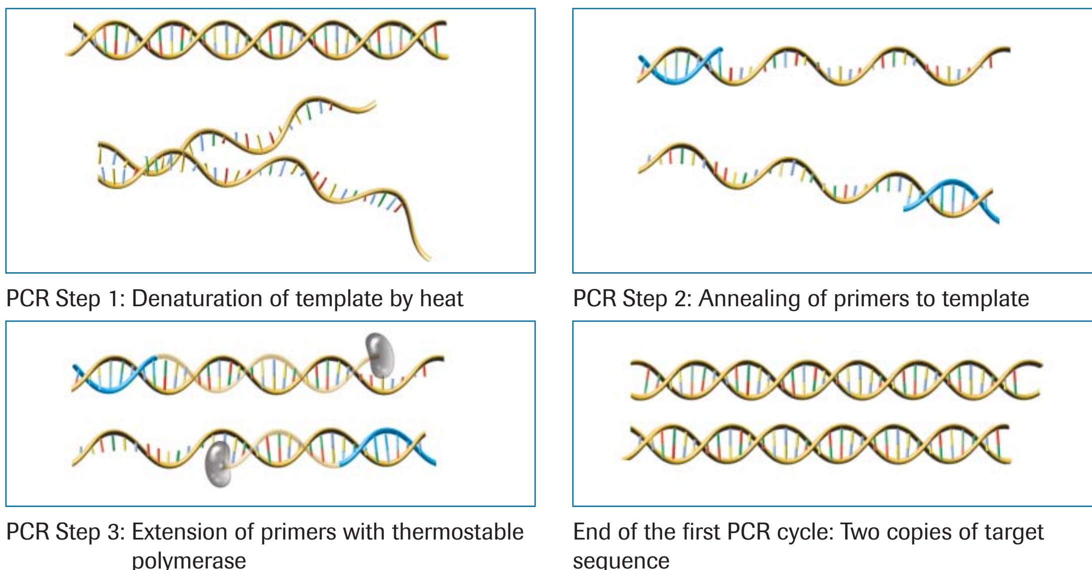
An Introduction to PCR

Twenty years ago, the Polymerase Chain Reaction (PCR) - an amazingly simple idea - was described as the revolutionary scientific technique of the 20th century (see VanGuilder, H.D. *et al.* (2008). "Twenty-five years of quantitative PCR for gene expression analysis." *Biotechniques* **44**(5): 619-26).

The reason for its extraordinary success is that PCR solved a fundamental problem biologists faced at that time - how to perform *de novo* DNA synthesis *in vitro*, thus allowing to amplify any desired target sequence, the original concentration of which, in most cases, is too small for downstream examination. Basically, PCR is an enzymatic DNA amplification process, mimicking to some extent *in vivo* replication, divided into a series of cycles. Theoretically, if optimal reaction conditions exist, every cycle of the PCR process doubles the amount of the desired DNA fragment available, resulting in exponential product accumulation. If enough molecules have been newly synthesized, the amplicons can be visualized by means of fluorescent dyes. Impressively, the PCR technique is so sensitive that it can detect just one molecule in a complex DNA sample.

Within a few years, the Polymerase Chain Reaction took the world's biological laboratories by storm and became the key technology in the field of molecular biology. The generation of virtually unlimited amounts of specific PCR products made many new methods of DNA analysis possible, such as downstream modifications (*e.g.*, cloning of the PCR product to establish gene libraries) or comprehensive product analysis techniques (*e.g.*, gene expression or genotyping analysis).

It all started in 1983 with an idea by Kary Mullis, who worked as a scientist for Cetus, a small, innovative California biotech company. Modestly he recounts the "combination of coincidence, naiveté and a series of lucky errors," which led him to the PCR technique, this captivatingly simple method of effectively mass-duplicating DNA. PCR reaped the highest scientific honor for its inventor in record time. In 1993, just ten years after his brilliant idea, Kary Mullis received the Nobel Prize for Chemistry.



PCR Step 1: Denaturation of template by heat

PCR Step 2: Annealing of primers to template

PCR Step 3: Extension of primers with thermostable polymerase

End of the first PCR cycle: Two copies of target sequence

Figure 1: The PCR Principle. PCR is a cyclic DNA amplification process. Each cycle involves three steps, which are described in detail above.

Real-Time PCR

The invention of Real-Time PCR technology represented another revolutionary leap forward in the field of DNA analysis. This advanced version of the conventional original PCR method makes powerful new qualitative and quantitative DNA analysis possible, in addition to highly sensitive DNA amplification. The key feature of this innovative PCR technique is the ability to monitor the DNA amplification process as it happens. Both the enzymatic PCR process itself and detection of the PCR product occur in the same reaction vessel.

Monitoring Real-Time PCR requires dedicated instrumentation capable of collecting data online from every cycle. Real-Time PCR instruments measure PCR product accumulation by means of dyes that fluoresce in the presence of DNA. This may be accomplished directly with intercalating dyes, which bind to the double stranded DNA (*e.g.*, SYBR Green I), or indirectly with fluorescence-labeled oligonucleotides (commonly known as fluorescent probes), which recognize and bind by base pairing to a specific region or site on one DNA strand. Measurement of fluorescence at every PCR cycle allows the product accumulation to be displayed on a plotted curve. The amount of fluorescence is proportional to the amount of PCR product in the reaction vessel. If a specific target DNA is present in a sample, the time it takes (or number of cycles required) to generate a detectable signal depends on its initial concentration in the sample. Use of this cycle number information thus enables easy qualitative and quantitative gene analysis.

Higuchi and co-workers at Roche Molecular Systems and Chiron initially demonstrated Real-Time PCR (Higuchi, R., C. Fockler, et al. (1993). "Kinetic PCR analysis: real-time monitoring of DNA amplification reactions." *Biotechnology* **11**(9): 1026-30.). By including a common fluorescent dye (ethidium bromide, EtBr) in the PCR mix and running the reaction under ultraviolet light (which causes EtBr to fluoresce), they were able to visualize and record the accumulation kinetics of PCR products. This powerful new technology quickly matured, dramatically simplifying the recognition and analysis of amplicons.

With Real-Time PCR and especially the Roche Applied Science LightCycler® Systems, minimal amounts of DNA can be replicated very rapidly and detected easily.

The LightCycler® Carousel-based System (LightCycler® 1.5 and 2.0 Instruments) has repeatedly set new standards for Real-Time PCR since its first introduction in 1997. It was the first system to support hybridization probes, variant identification based on the automated grouping of melting curve profiles, automated absolute quantification, and relative quantification with efficiency correction (E-method). First presented in 2005, the LightCycler® 480 Real-Time PCR System extends LightCycler® System accuracy, speed, and versatility to medium- and high-throughput applications in gene expression and genotyping analysis.

Figure 2: LightCycler® Carousel-based Instruments and LightCycler® 480 Instrument.

Principles of Kinetic PCR

Real-time instruments allow researchers to follow the amplification of DNA molecules and – indirectly via fluorescence measurements – observe the accumulation of PCR products as it happens. Since the entire course of DNA amplification in each vessel is monitored, Real-Time PCR is also called kinetic PCR. The kinetics of PCR amplification in a single sample, if plotted on a graph (number of amplified molecules against number of cycles), has a sigmoid profile resembling a bacterial growth curve (see Figure 3 below). The reality seems to contradict the theory, but it agrees with the fact that PCR is an enzymatic process. Every enzymatic process can be described by a sigmoidal curve profile, which results from processes in which a period of maximum enzymatic activity is followed by a period of reduced activity (called the plateau phase).

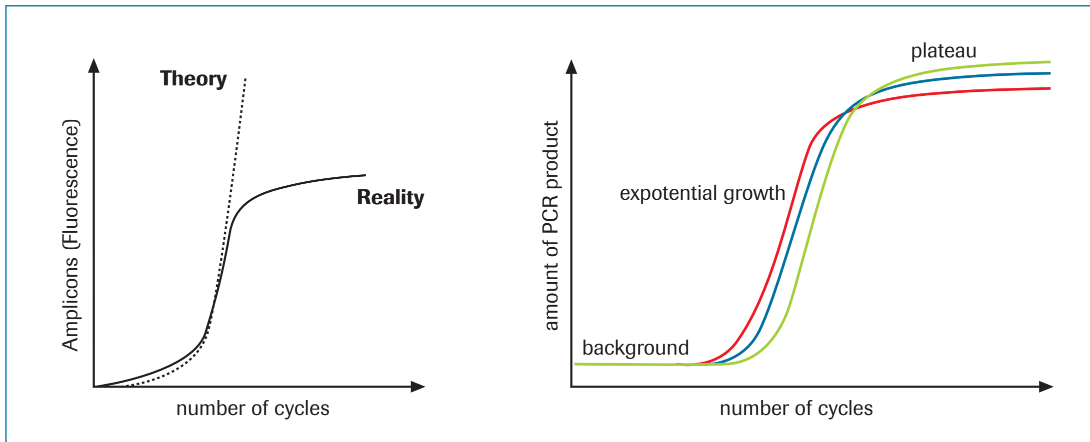


Figure 3

Detailed analysis of the PCR kinetic profile reveals that the curve may be divided into three characteristic phases: the early background phase, the middle exponential growth phase (or log linear phase), and the late plateau phase.

The early PCR phase is characterized by background fluorescence (shaded area in Figure 4, next page) and represents the part of the PCR that cannot be seen with any modern technique. With every PCR cycle the product amount increases until its fluorescence intensity exceeds background. The PCR cycle at which product fluorescence intensity finally rises above background and becomes visible is called the crossing point (Cp). At this point in the PCR, there are approximately 10^{10} to 10^{12} amplified molecules in the reaction tube. Quantification with Real-Time PCR is performed at this stage of the reaction.

The exponential PCR phase begins as the product fluorescence rises above the background. Theoretically, the rate of product amplification is maximal during this part of the reaction; hence the efficiency of this part of the reaction equals two and the amount of product doubles in every cycle under optimal operating conditions.

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Finally, late in the PCR, the reaction enters the plateau phase, which is characterized by lower product accumulation rates and continuously varying efficiency. The observed decrease in enzymatic activity during the late phases of PCR may be caused by steadily changing reaction conditions (*e.g.*, reduced concentrations of substrates such as dNTPs), increasing accumulation of by-products (*e.g.*, pyrophosphate), increases in competitor (side) reactions, or re-annealing of amplification product.

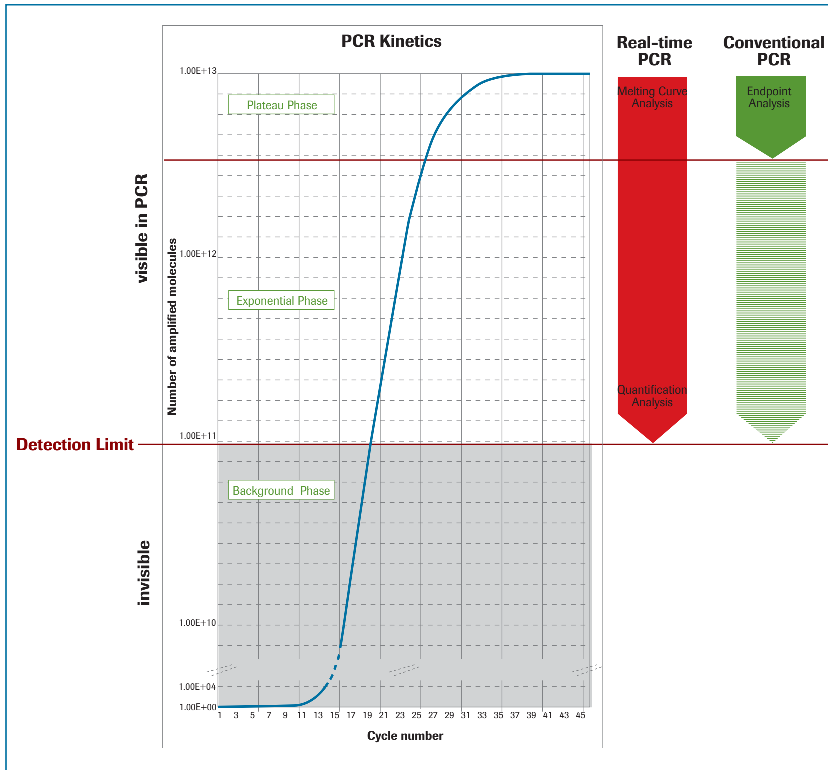


Figure 4

For theoretical considerations on PCR, the exponential phase is often divided further into two subsections: a short section above the background phase (the "exponential amplification phase") and a subsequent longer part (the "linear amplification phase," which is defined by a steep increase in the fluorescence signal).

Acquisition of Fluorescence Signal

Cycle-by-Cycle Measurement

Only the early exponential phase can be used for quantification of initial sample input, because only this phase follows predictable and optimal kinetics.

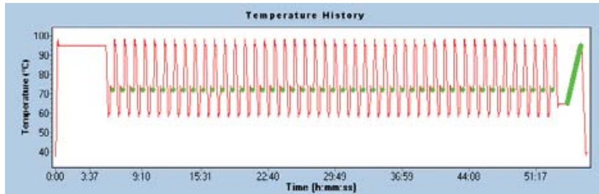


Figure 5: Overall temperature profile of a typical PCR reaction.

In Real-Time PCR, data analysis makes use of the time (or PCR cycle) at which the target amplification is first detected. This value, known as Cp, is representative of the initial exponential phase. There is a correlation between Cp and concentration: the higher the concentration of target nucleic acid in the starting material, the sooner a significant increase in fluorescent signal will be observed, yielding a lower Cp. This correlation between amount of template and value of Cp facilitates all types of Real-Time PCR-based quantitative analysis.

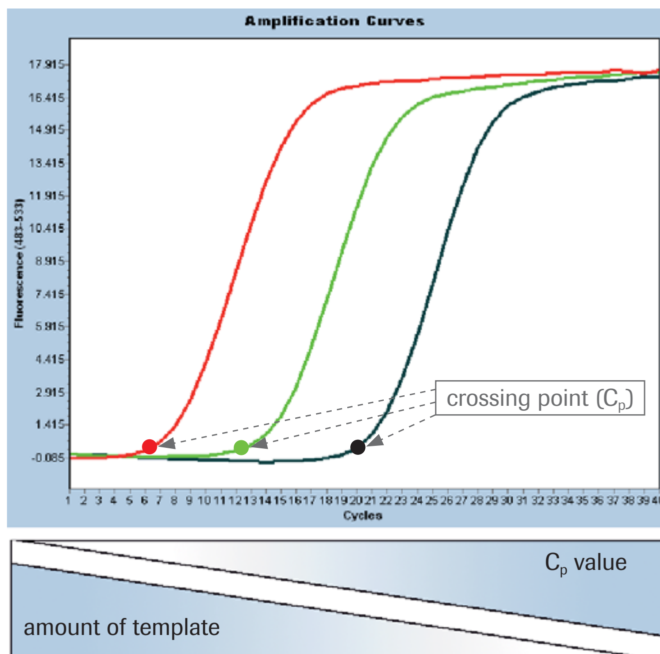


Figure 6: Amplification curves in Real-Time PCR; correlation between C_p and concentration.

Because of this relationship between Cp and starting concentration, Real-Time PCR makes data acquisition and analysis during the exponential phase easy and therefore allows sensitive quantification of a given target. Furthermore, the data gathering capabilities of Real-Time PCR instruments make it possible to detect DNA over a broad range of concentrations - typically more than ten orders of magnitude.

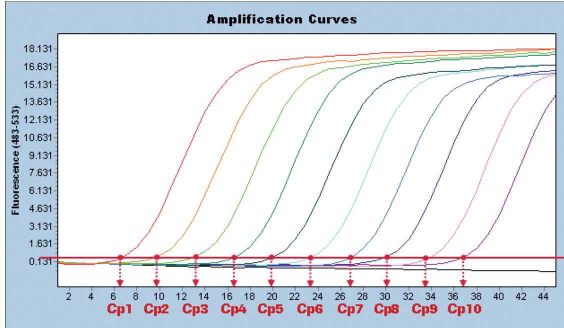


Figure 7: Real-Time PCR dynamic range.

In addition to visualization of the amplification process, Real-Time PCR instruments provide several different methods of qualitative (*e.g.*, bacterial identification) and quantitative (*e.g.*, gene expression) analysis. During the last decade, Real-Time PCR instrument manufacturers have put great effort into improving the analytical capabilities of the instrument software.

Since PCR amplification and detection takes place in the same tube, Real-Time PCR techniques no longer require the cumbersome and time-consuming post-PCR analysis steps associated with conventional PCR. Thus, one source of contamination and variability in results is eliminated in qualitative and quantitative Real-Time PCR analysis.

Melting Curves

Real-Time PCR provides results based not only on the amplification reaction (qualitative detection and quantitative analysis), but also on additional data generated during post-amplification melting curve experiments. These additional results are highly useful for DNA product characterization.

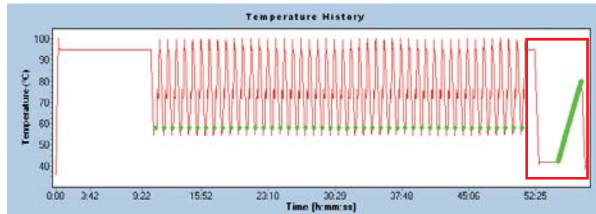
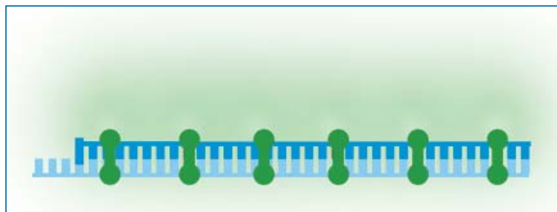


Figure 8: Temperature profile of post-PCR melting curve analysis (red box).

For post-PCR product characterization, PCR products are melted as the reaction chamber temperature is slowly increased (from approx. 45°C to 95°C). During the melting process the fluorescence signal decreases, due to the release of bound dye molecules as double-stranded DNA molecules become single-stranded. Changes in the fluorescence signal are continuously monitored. The resulting melting curve is usually converted to a "first derivative" plot for easier analysis (-dF/dT plotted as a function of T). Each product in an amplicon mixture can be characterized by its specific melting point, which depends mainly on GC content and length of the amplicon sequence. In summary, this Real-Time PCR procedure for amplification product characterization makes post-PCR handling steps like agarose gel electrophoresis unnecessary and reduces both intra- and inter-assay variability of results.



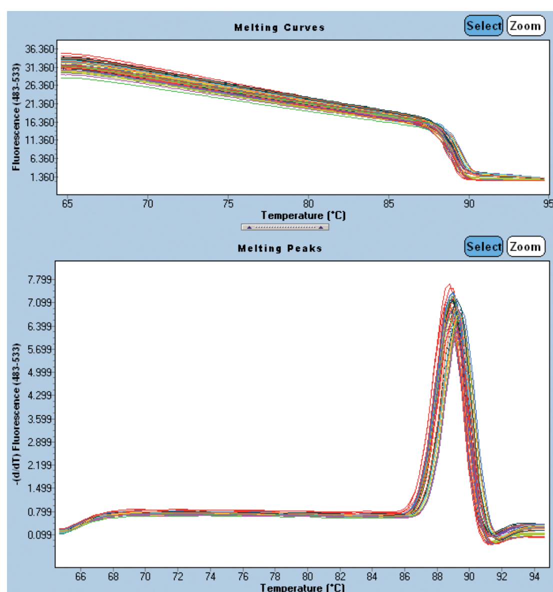


Figure 9: Use of SYBR Green I dye for PCR product characterization.

Another group of melting curve assays rely on the sequence-specific binding of fluorophore-labeled oligonucleotide probes which bind to complementary sequences in targets. This specificity of interaction between target and probes can be used to extract even more information on the amplicon´s characteristics. For instance, if a SNP (single nucleotide polymorphism) exists in the region that binds to the probe, the probe-target hybrid is destabilized and melts at a lower temperature than a hybrid between the probe and the perfectly matching sequence, thus allowing this technique to be used for genotyping (SNP detection).

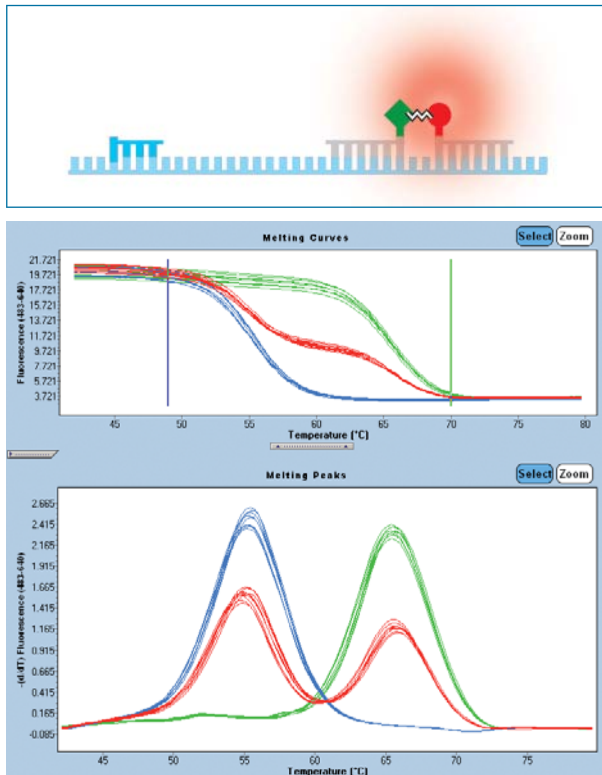
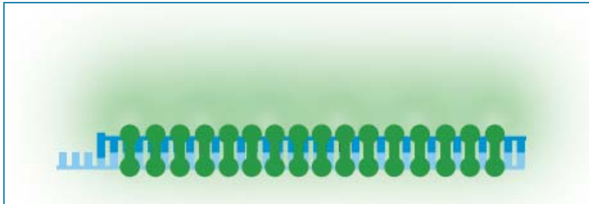


Figure 10: Melting curve analysis with fluorescence-labeled probes.

High Resolution Melting (HRM), a third melting curve analysis method, is a more recently developed refinement of the thermal dissociation principle. For initial detection of sequence variations, differences in the melting curves of the amplicons are analyzed, using special unspecific, highly saturating fluorescent dyes instead of probes. DNA from heterozygote samples forms heteroduplexes that begin to separate into single strands at a lower temperature and with a different curve shape than DNA from homozygotes. In many cases, depending of course on the specific sequences present, homozygote samples differing in only one base pair (*e.g.*, wild-type and mutants,) can be differentiated with this method, too.



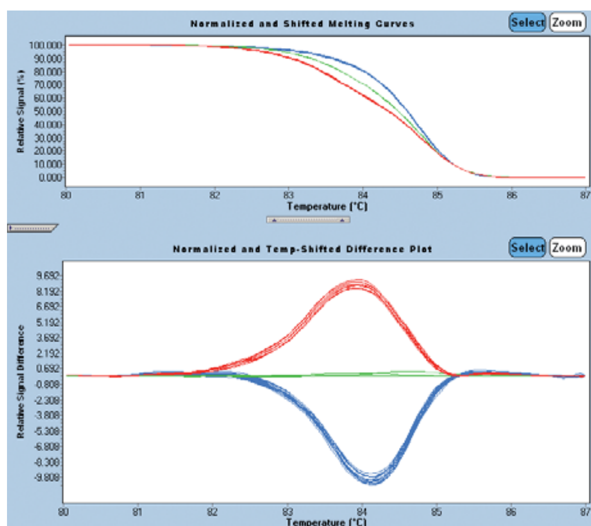


Figure 11: HRM analysis with non-specific, saturating DNA dyes allows differentiation of homo- and heterozygotes.

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Detection Formats

All Real-Time PCR systems detect PCR products by means of fluorescent dyes. There are several dye-based methods used in product detection, characterization or quantification. Dyes can either be used as such and just added to the PCR master mixes, or attached covalently to oligonucleotides used as sequence –specific probes. The resulting, most commonly used assay formats - SYBR Green I, HybProbe probes, and hydrolysis probes - are described in the following.

SYBR Green I

SYBR Green I is a dye that binds to all double-stranded DNA molecules, regardless of sequence. When SYBR Green I dye intercalates into dsDNA, its fluorescence increases significantly. During the different stages of PCR, the intensity of this fluorescence will vary, depending on the amount of dsDNA present.

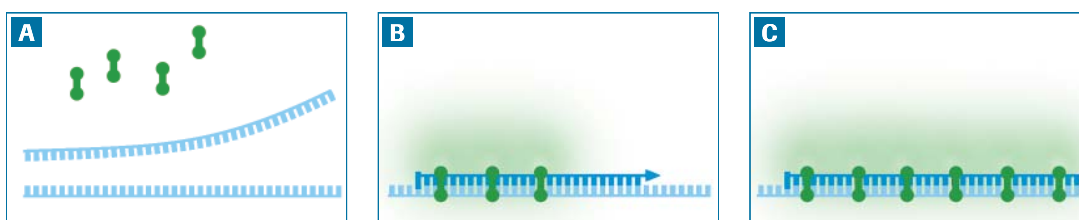

Figure 12: PCR in the presence of SYBR Green I.
SYBR Green I dye only fluoresces when it is bound to double-stranded DNA (dsDNA) and excited by blue light. SYBR Green I does not bind to single-stranded DNA, so fluorescence is minimal during denaturation. As dsDNA forms **(panel A)** and is synthesized **(panel B)**, SYBR Green I binds the dsDNA and the fluorescent signal from the bound SYBR Green I (green light) increases. At the end of elongation **(panel C)**, all DNA is double-stranded, the maximum amount of SYBR Green I is bound and the fluorescent signal is at its maximum for that PCR cycle. Therefore, the fluorescent signals from SYBR Green I are measured at the end of each elongation phase.

LightCycler® 480 ResoLight Dye

LightCycler® 480 ResoLight Dye belongs to a new generation of dsDNA-binding dyes that can detect the presence of heteroduplexes formed during PCR (*e.g.*, if the sample is heterozygous for a particular mutation). Its possible applications therefore exceed those of other, more traditional DNA-staining dyes like SYBR Green I.

LightCycler® 480 ResoLight Dye is not toxic to amplification enzymes. Thus, high concentrations of the dye do not affect the PCR. These high concentrations completely saturate the dsDNA in the sample. Therefore, when dye molecules dissociate from dsDNA during melting, there is only little chance for them to re-bind to other unoccupied sites. This makes the melting process highly homogeneous and the acquired signals very sharp. Under these conditions, even small changes in the melting curve result in subtle, but reproducible changes in fluorescence.

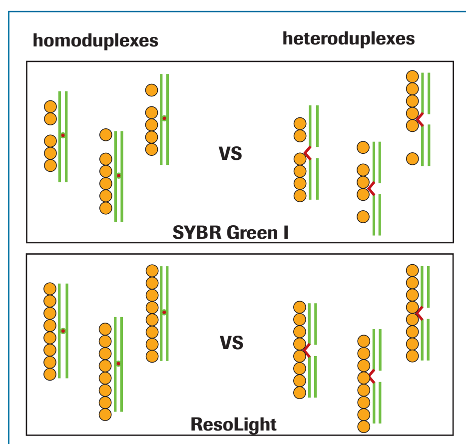

Figure 13: ResoLight dye - unlike SYBR Green I - does not make dye/DNA complexes form or melt differently. Only sequence differences (full match or mismatch) change melting behaviour, making them detectable with this dye.

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Principles of Specific Probe Assays

HybProbe - and hydrolysis probe assays are sequence-specific techniques, relying on oligonucleotide probes that hybridize to their complementary sequence in the target PCR product and thus only detect this specific product. Both formats take advantage of the FRET principle (Fluorescence Resonance Energy Transfer). Whereas HybProbe probes emit fluorescence via FRET when they bind target, the fluorescence of hydrolysis probes is quenched by FRET in the unbound state and they must be hydrolyzed by Taq DNA polymerase after binding to the target sequence to emit fluorescence.

HybProbe Probes

The unique LightCycler® HybProbe format is based on the FRET principle. Two sequence-specific oligonucleotide probes are labeled with different dyes (donor and acceptor), and are added to the reaction mix along with the PCR primers. During the annealing phase, HybProbe probes hybridize to the target sequences on the amplified DNA fragment in a head-to-tail arrangement, thereby bringing the two dyes into close proximity.

The donor dye (fluorescein) is excited by blue light at ~470 nm. When the two dyes are close to each other (within 1-5 nucleotides), the energy emitted by the donor dye excites the acceptor dye attached to the second HybProbe probe, which then emits fluorescent light at a different wavelength. The amount of fluorescence is directly proportional to the amount of target DNA generated during the PCR process. HybProbe probes are displaced, but not cleaved, during the elongation and denaturation steps.

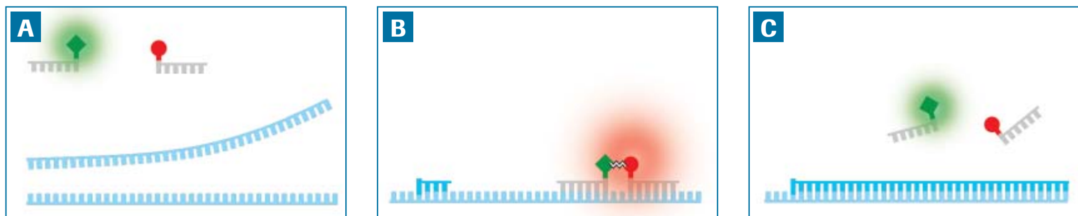

Figure 14: Principle of target detection by HybProbe probes.
Panels A-C show the behavior of HybProbe probes during different stages of PCR. In the example shown, the donor dye probe has a fluorescein label at 3' end, and the acceptor dye probe has a LightCycler® Red label at its 5' end (note that this setup might also be reversed, *i.e.*, donor dye at the 5' and acceptor dye at the 3' end). Hybridization does not occur during the denaturation phase of PCR **(panel A)**. Since the distance between the unbound dyes prevents energy transfer, no fluorescence will be emitted by the red acceptor dye during this phase. In panel B, the probes hybridize to the amplified DNA fragment in a head-to-tail arrangement, thereby bringing the two fluorescent dyes close to each other. Fluorescein is exited by blue light which causes it to emit green fluorescent light. The emitted energy excites the LightCycler® Red dye. After annealing, an increase in temperature leads to elongation and displacement of the probes. At the end of the elongation step, the PCR product is double-stranded, while the displaced HybProbe probes are back in solution and too far apart to allow FRET to occur **(panel C)**. The red fluorescence emitted by the second probe is measured at the end of each annealing step **(panel B)**, when the fluorescence intensity has reached its maximum.

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Hydrolysis Probes

Hydrolysis probe assays, conventionally called TaqMan® assays, can technically be described as homogenous 5´ nuclease assays, since a single 3´ non-extendable hydrolysis probe, which is cleaved during PCR amplification, is used to detect the accumulation of a specific target DNA sequence. This single probe contains two labels, a fluorescence reporter and a fluorescence quencher, in close proximity to each other. When the probe is intact, the quencher dye is close enough to the reporter dye to suppress the reporter fluorescent signal (fluorescence quenching takes place via FRET). During PCR, the 5´ nuclease activity of the polymerase cleaves the hydrolysis probe, separating the reporter and quencher. In the cleaved probe, the reporter is no longer quenched and can emit a fluorescence signal when excited.

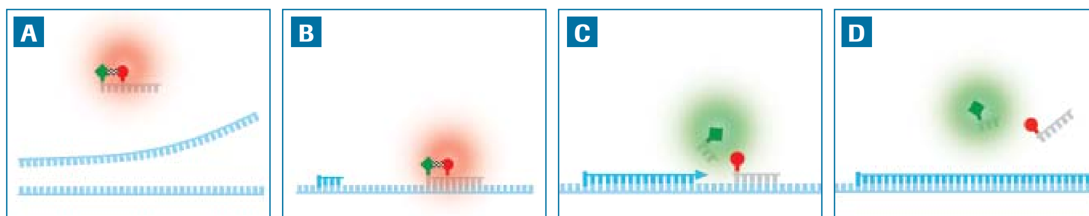

Figure 15: Schematic diagram of the Hydrolysis Probes format.
Panels A-D show the behavior of hydrolysis probes during PCR. The probe carries two fluorescent dyes in close proximity, one of which (quencher) quenches the fluorescence from the other (reporter) as long as the probe is intact. In the denaturation phase (**panel A**), the strands of the target DNA separate as the temperature increases. During the annealing phase (**panel B**), primers and probes specifically anneal to the target sequence (the hydrolysis probe is phosphorylated at the 3' end, so it cannot be extended). As the DNA polymerase extends the primer, the 5' nuclease activity of the enzyme will cleave the probe (**panel C**), allowing the reporter dye to emit green fluorescence. The probe fragments are then displaced from the target and polymerization of the new amplicon continues (**panel D**). The DNA polymerase will separate the reporter and quencher only if the probe has hybridized to the target. Accumulation of PCR products is detected directly by monitoring the increase in green fluorescence from the reporter dye. The fluorescent signal of the reporter dye is measured at the end of each elongation phase (**panel C**).

Universal ProbeLibrary (UPL)

UPL probes are a special type of hydrolysis probes. These short LNA-modified probes (8-9mers; 165 pre-designed UPL probes in total) detect a specific PCR amplicon, but also bind to more than one site in the transcriptome. However, their combination with suitable target-specific primers results in a target-specific assay. Primers and probes for UPL assays can both be easily designed and selected using the free, web-based Assay Design Center (*for more information, see "Recommendations for Using the Universal ProbeLibrary" in chapter 3,* ***Assay Development: Tips and Tricks***).

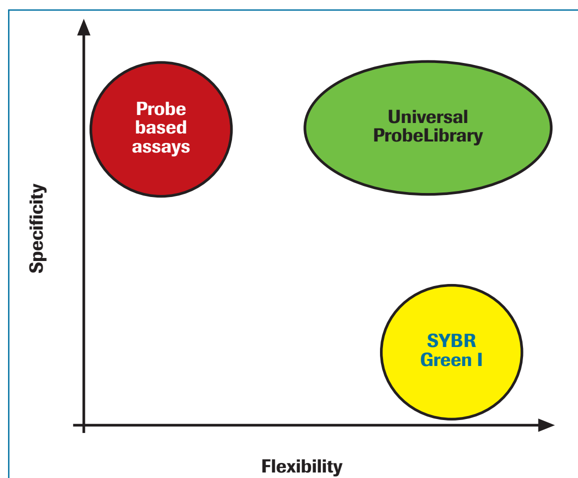

Figure 16: UPL assays represent a unique combination of specificity and flexibility.

SimpleProbe Probes

SimpleProbe probes are a special type of hybridization probes. They differ from HybProbe probes in one important way: instead of two probes per assay, only one single probe carrying a covalently attached dye is needed. This single probe hybridizes specifically to a target sequence. Once hybridized, the SimpleProbe probe emits more fluorescence than it does when it is not hybridized to its target. As a result, changes in fluorescence depend solely on the hybridization status of the probe.

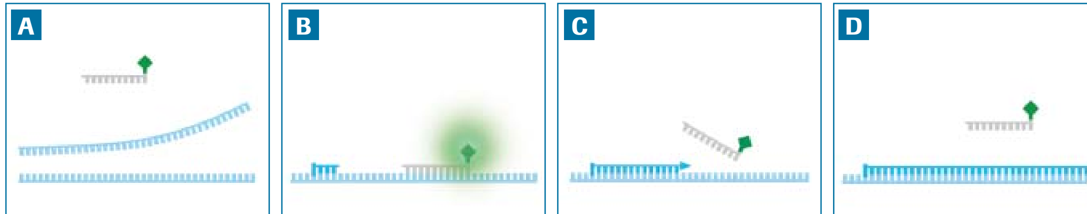

Figure 17: Schematic diagram of the SimpleProbe format.
During the denaturation phase no hybridization takes place, thus only a low fluorescence background is detected at 530 nm (**panel A**). During the annealing phase, the probe hybridizes to the amplified DNA fragment and is no longer quenched. Fluorescein, when excited by the LightCycler® LED, emits green fluorescent light which is measured only at the end of each annealing step at maximum intensity (**panel B**). During the subsequent elongation step, the SimpleProbe probe is displaced (**panel C**). At the end of the elongation step, the PCR product is double stranded and the displaced SimpleProbe probe is again quenched (**panel D**).

Other Formats

Other formats, *e.g.,* molecular beacons, can also be used on Roche Real-Time PCR Systems if the dyes on the probes are compatible with the available channels and filters of the respective LightCycler® Instrument.

Scorpions® probes

Scorpions® provide a good example of an assay format that can be implemented on LightCycler® Systems. They consist of PCR primers that contain a probe with a "stem-loop" tail, a fluorophore, and a quencher. The "stem-loop" tail is separated from the PCR primer sequence by a "PCR blocker", a chemical modification that prevents the Taq DNA polymerase from copying the stem-loop sequence of the Scorpions® primer. Scorpions® differ from the specific detection methods discussed above in that their mechanism of detection is by intramolecular primer extension. During PCR, Scorpions® primers are extended to form PCR products. During the annealing phase, the probe sequence in the Scorpion's tail curls back to hybridize to the target sequence in the newly formed PCR product. As the tail of the Scorpions® and the PCR product are now part of the same strand, the interaction is intramolecular, kinetically favorable and highly efficient.

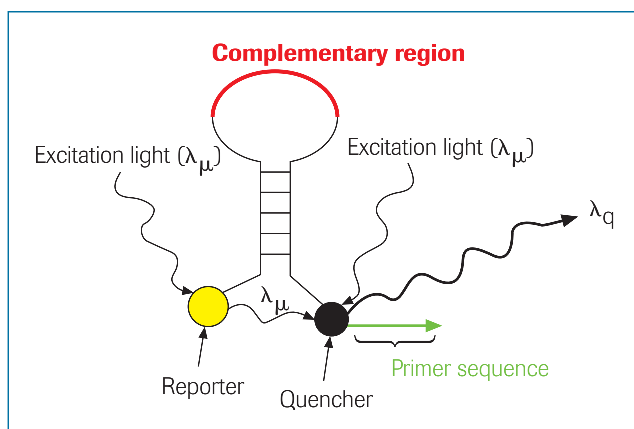

Figure 18: Schematic depiction of the Scorpions® probe format.

1

Multiplexing

The simplest Real-Time PCR assays involve one target analyzed with one type of dye or probe. More complex PCR assays generate one amplicon (monoplex PCR), but detect several targets on that amplicon. Each of these targets is detected with a differently labeled colored probe (multicolor detection). Even more complex assays involve multiplex PCR, which not only have multiple detection sites, but include more than one primer set in the reaction (multiplex PCR), thus allowing two or more different targets to be amplified simultaneously. Typically, a multiplex PCR detects the target gene of interest in one channel while a control gene is detected in another channel (duplex PCR with internal control).

Xenon lamp (430-630							
Dye	LightCycler® Cyan 500	SYBR Green I ResoLight	Fluorescein FAM	HEX VIC	LightCycler® Red 610	LightCycler® Red 640	Cy5

Figure 19: Overview of different fluorophores recommeded for use on the LightCycler® 480 Instrument

The LightCycler® 480 Instruments I and II are ideally suited for multiplex PCR (*e.g.,* amplifying and detecting up to 5 templates in one well). All LightCycler® 480 Instruments employ the high-intensity LightCycler® 480 Xenon Lamp that emits light over a broad wavelength range (430-630 nm), which enables the instrument to detect probes labeled with many different fluorophores (see Figure 19 for an overview; for more detailed information on the optimal choice of dye and detection channel combinations, please refer to the operator´s manual of your respective LightCycler® 480 System).

Applications

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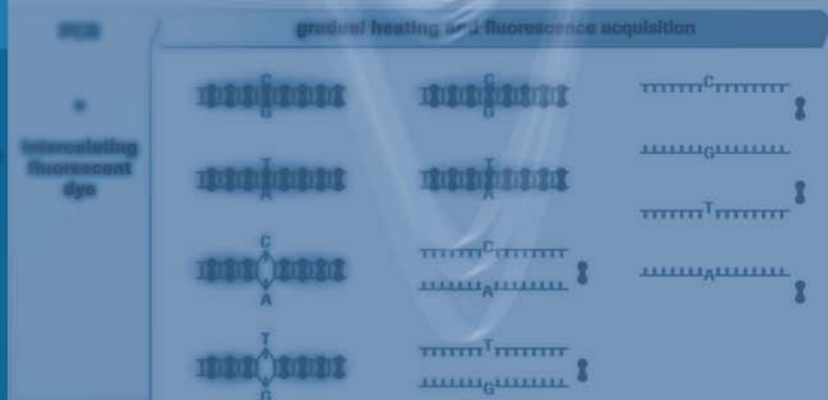
AAGCACGGATTCCA
AAGCACGAATTCCA



Gene Detection

Qualitative Analysis

The most basic form of genetic analysis consists in assays that require only a “yes/no” answer to the question whether a gene is present in a sample. Such qualitative approaches detect the presence of a particular target in unknown samples, based on whether the target is amplified or not. The analysis does not quantify the amount of target DNA, thus standards are not required. A qualitative detection analysis can be performed on any experiment containing an amplification program and data.

Although not strictly required, both positive and negative control samples should be included in these qualitative assays. An even more complex experimental setup includes an internal control and requires a dual color approach (*see Absolute Quantification with External Standards and Internal Control, page 27*).

In the LightCycler® Software 4.05/4.1 (running on carousel-based LightCycler® Instruments) a control concept is available to automatically check calls of all unknown samples as valid or invalid.

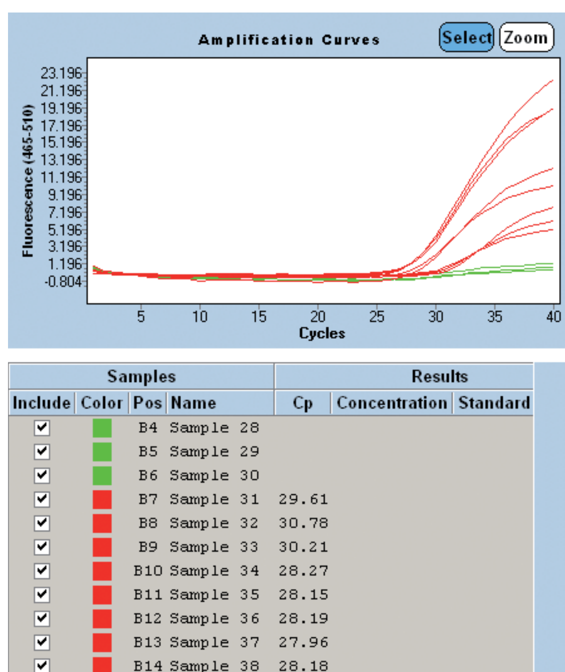


Figure 20: Qualitative detection analysis performed on a carousel-based LightCycler® Instrument using LightCycler® Software 4.05.

Quantification

Overview of Quantitative PCR Methods

There are many types and subtypes of quantitative Real-Time PCR methods, each of which is characterized by its requirements, its complexity, and its reliability. However, it is possible to group all these methods under two main analysis techniques - absolute and relative quantification. The technique you choose depends on the complexity of your analysis and the form of the final result:

- **Absolute quantification** allows you to quantify a single target sequence and express the final result as an absolute value (*e.g.*, viral load in copies/ml). Such analyses routinely occur in research areas like virology and microbiology.
- **Relative quantification** compares the levels of two different target sequences in a single sample (*e.g.*, target gene of interest [GOI] and another reference gene) and expresses the final result as a ratio of these targets.

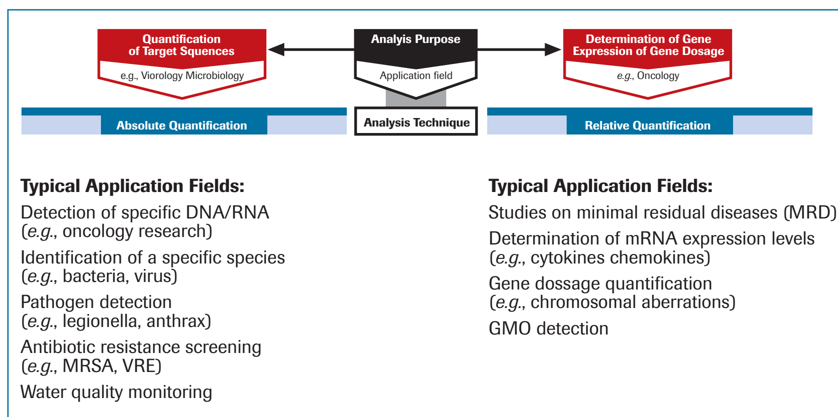
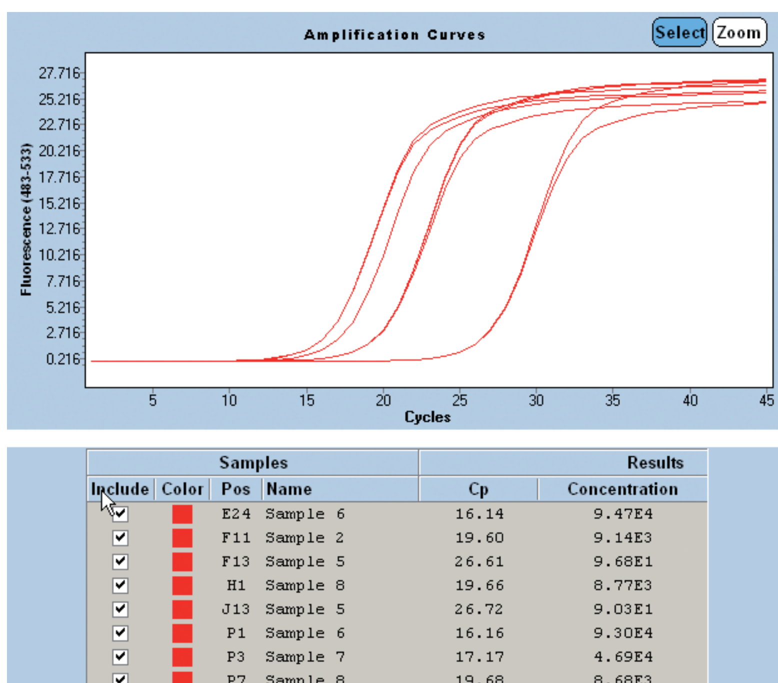


Figure 21: Typical application fields for absolute quantification and relative quantification.

Both methods take advantage of the correlation between fluorescence and the amount of PCR product accumulating during PCR. The crossing point (Cp), the cycle at which fluorescence intensity exceeds background, represents the same amount of PCR product in every curve. Therefore the initial template concentration in different samples can be correlated to real-time fluorescence curves by comparing the crossing points of each sample. In other words, the samples with the highest number of initial template copies before the onset of PCR reach the crossing point the earliest.



Samples				Results	
Include	Color	Pos	Name	Cp	Concentration
✓		E24	Sample 6	16.14	9.47E4
✓		F11	Sample 2	19.60	9.14E3
✓		F13	Sample 5	26.61	9.68E1
✓		H1	Sample 8	19.66	8.77E3
✓		J13	Sample 5	26.72	9.03E1
✓		P1	Sample 6	16.16	9.30E4
✓		P3	Sample 7	17.17	4.69E4
✓		P7	Sample 8	19.68	8.68E3

Figure 22

2

Absolute Quantification

Absolute Quantification with External Standards

The easiest way to obtain an absolute value for an unknown concentration of target is to compare the Cp of an unknown sample against those of standards with known copy numbers.

Standard material can be selected from various sources (*e.g.*, linearized plasmid DNA carrying the cloned target sequence, purified PCR products). The target concentration in the standard must be known. PCR is then performed with a series of dilutions of the standard, which represent different target concentrations in a reaction.

The known template amount of each dilution is automatically plotted against the measured Cp values. The resulting regression line is called the standard curve and shows the correlation between Cp and concentration. By comparing the Cp values from samples with unknown amounts of template to this standard curve, one can immediately determine the starting amount of template in each sample.

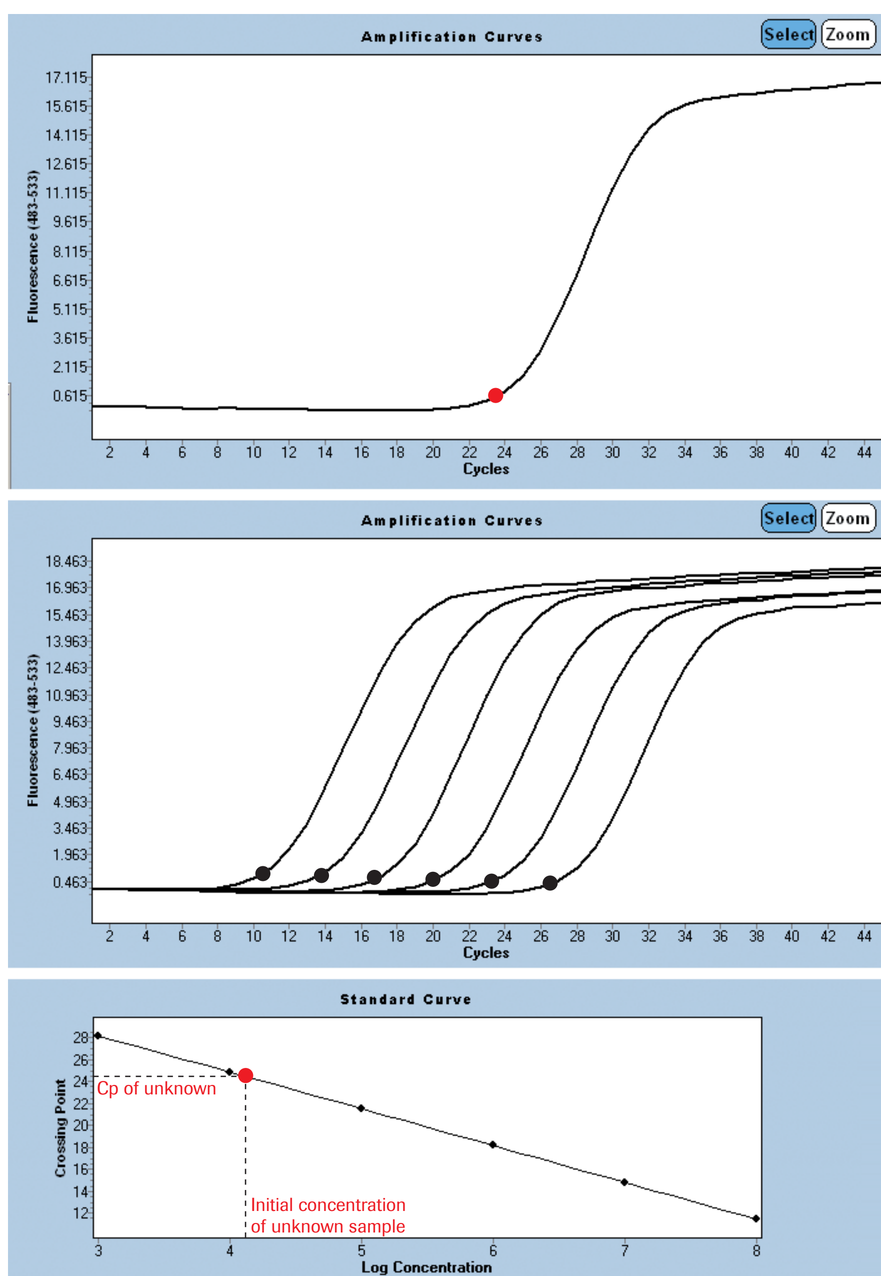


Figure 23: Absolute quantification with external standards.

Absolute Quantification with External Standards and Internal Control

An advanced analysis involves an exogenous control DNA, which is co-amplified with the target DNA in the same tube. Since each sample is spiked with a known amount of an internal control, this method requires dual color analysis (see *Multiplexing* in the *Detection Formats* chapter, page 22). In addition, the internal control must be amplifiable under the same PCR conditions as the target.

This internal control is mainly used to detect potential inhibitors in the sample material, which may affect PCR. In addition, if the control is added to the sample preparation earlier (*e.g.*, after the initial lysis of the biological sample material), it may also be used to monitor recovery during the nucleic acid (NA) isolation.

Such approaches, using internal controls, can easily distinguish between truly negative results and suppression due to a PCR inhibitor.

Figure 24: Absolute quantification with external standards and internal control. Green: standards; red: internal control; blue: unknown sample.

2

Relative Quantification

Relative Quantification

In many quantitative experimental studies, an absolute value for the sample under investigation is not required or, in many cases, cannot be obtained. Therefore, the analysis result is expressed as a relative expression value instead.

Relative quantification is commonly used in applications that need to compare changes in multiple samples even though the samples vary in quality or quantity. Such analyses are useful, for instance, in oncology research.

Concept of Relative Quantification

Relative quantification compares the levels of two different target sequences in a single sample (*e.g.*, target gene of interest and a reference gene) and expresses the final result as a ratio of these gene levels. However, the ratio of target gene to reference gene is a relative, dimensionless number that is meaningful only when compared between samples.

$$\text{relative ratio} = \frac{\text{concentration of target}}{\text{concentration of reference}}$$

Figure 25

The second, or reference, gene is a constitutively expressed gene or housekeeping gene that is found in constant copy numbers under all tested conditions. This reference gene, also known as an endogenous control, provides a basis for normalizing sample-to-sample differences.

For example, gene expression studies usually try to determine the way a target gene changes its expression profile over time (*e.g.*, how much the expression changes in the course of a disease or treatment) relative to a defined starting point (*e.g.*, disease-free or untreated state). Since relative quantification allows researchers to easily compare the expression levels of a target gene under at least two conditions (*e.g.*, disease-free / diseased or untreated / treated), relative quantification is the best technique for determining gene expression and gene dosage.

Planning Relative Quantification Assays

Gene expression assays must be accurate enough to quantify subtle changes in amounts of nucleic acid against a complex background. Furthermore, they must be reproducible enough to generate reliable data that can be compared over a long period of time or between different experimental systems. Both requirements are easily met by relative quantification assays on the LightCycler® Systems. However, if the goal is to produce accurate and reproducible results, the relative quantification assay must be carefully planned and designed to meet the needs of the experimental study.

Major steps to successful relative quantification assays (*e.g.*, for gene expression studies) include:

- Identification of the target gene(s) of interest (GOI) and suitable reference gene(s).
- Selection and preparation of sample material, including a so-called calibrator sample.
- Optimization of reaction conditions.

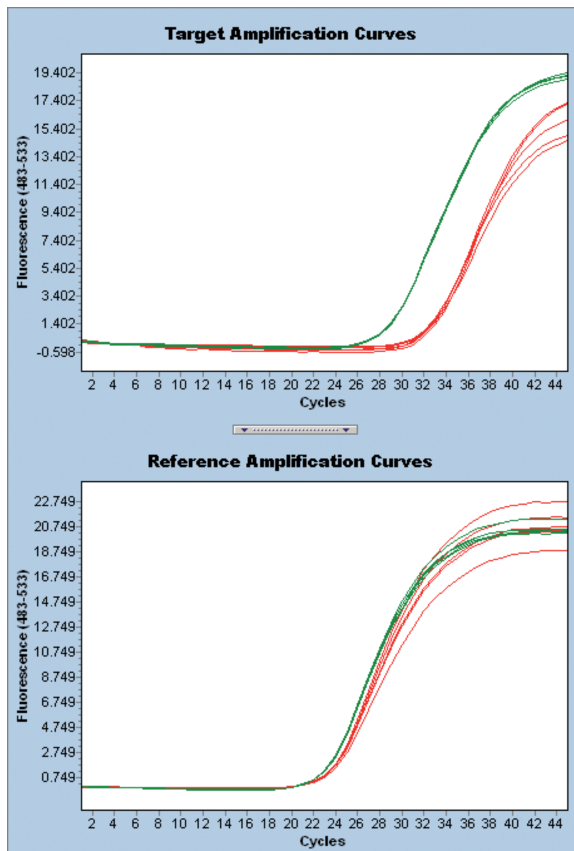


Figure 26

2

Reference Genes

Carefully select reference gene(s) for accurate normalization. To identify housekeeping genes that are reference gene candidates, we recommend searching the most recent articles in the literature for genes that are stably expressed in the biological system you study, and to choose primers and probes that recognize the mRNA (not only DNA) sequence of these genes. Consider genes from a wide variety of cellular processes.

Expression levels of housekeeping genes should not be affected by different experimental conditions. For any relative quantification, consider running comparative assays to demonstrate that the expression of these genes remains constant under all experimental conditions or, alternatively, to determine whether any observed variations in expression are significant.

If no single reference gene is suitable for all conditions, consider using more than one housekeeping gene and averaging their assay levels to form a single reference value.

Gene	Genomic structure / pseudogenes	Regulation *e.g.*
β-Actin	multigene family; > 20 genes; 1 active locus 20 pseudogenes	↑: hormones of tyroid gland ↑: stomach tumor
γ-Actin	multigene family; pseudogenes	
GAPDH	multigene family; 10-30 genes; > 200 in mouse mostly pseudogenes	↑: lung, pancreatic, colon cancer ↑: Insulin, EGF
5.89, 189, 289 RNA	pseudogenes	
β2-microglobulin	no pseudogenes	↑: Non-Hodgkin lymhoma abnormal expression in tumors
G6PDH	no pseudogenes growth factors	↑: kidney, stomach tumor ↑: hormones, oxydant stress
PBGD	no pseudogenes	
Aldolase	pseudogenes	
HPRT	pseudogenes	
U3, U8	pseudogenes	
Ornithin Decarboxylase		↑: tumors

Figure 27: Reference Genes

Normalization to a reference gene corrects for qualitative and quantitative differences in the sample, such as those caused by:

- Variations in initial sample amount or nucleic acid recovery
- Possible RNA degradation in sample material
- Differences in sample and/or nucleic acid quality
- Variations in cDNA synthesis efficiency
- Variations in sample loading or pipetting errors
- PCR inhibitors and other factors influencing PCR

Calibrator Sample

The relative amount of a target gene in an unknown sample is a unitless number that is only meaningful when compared to the relative amount of the gene in other samples. The most common way to make this comparison is to designate one of the samples as a so-called calibrator. Then, all other samples are compared to the calibrator.

For normalization of the final results, the target/reference ratio of each sample is divided by the target/reference ratio of the calibrator sample (the normalized value of the calibrator is 1).

The calibrator sample usually has a stable ratio of target gene expression to endogenous reference gene expression. This calibrator – typically a positive control sample - might be *e.g.*, RNA isolated from a certain cell line or tissue. Other examples of a typical calibrator sample are:

- An untreated cell line
- Sample at the time point “zero“
- Normal tissue

2

$$\text{calibrator normalized ratio} = \frac{\dfrac{\text{concentration of target}}{\text{concentration of reference}}\ \text{(sample)}}{\dfrac{\text{concentration of target}}{\text{concentration of reference}}\ \text{(calibrator)}}$$

Figure 28

When the identical calibrator sample is included in every relative quantification experiment within a study, it provides a constant reference point between several experiments, thus correcting for run-to-run and lot-to lot differences. This so-called assay calibrator corrects not only for differences in detection sensitivity between target and reference gene, but also for differences between several experiments (*e.g.*, during long-term studies).

Be aware that in respect of calibrator more than one definition exists in the literature.

Assay Calibrator:
The same calibrator sample is included in every relative quantification experiment (in-run) within a study.

Study Calibrator:
The study calibrator is performed on a separate multiwell plate (reference experiment).

2

Optimization of Reaction Conditions

Specificity, sensitivity, efficiency, and reproducibility are the important criteria to consider when optimizing a quantitative assay. A well-designed gene expression assay will display little or no test-to-test variations in crossing point and fluorescent signal intensity. PCR must be optimized in order to generate robust assays that are not affected by the normal heterogeneity of template nucleic acid.

Proper planning of the PCR (selection of primers and/or probes with appropriate design, suitable purification methods) will markedly affect the quality of PCR and especially the quantification. The performance of Real-Time PCR assays can also be affected by changes in primer and probe concentration, cycling conditions and buffer composition. For details, refer to the ***Assay Development: Tips and Tricks*** chapter.

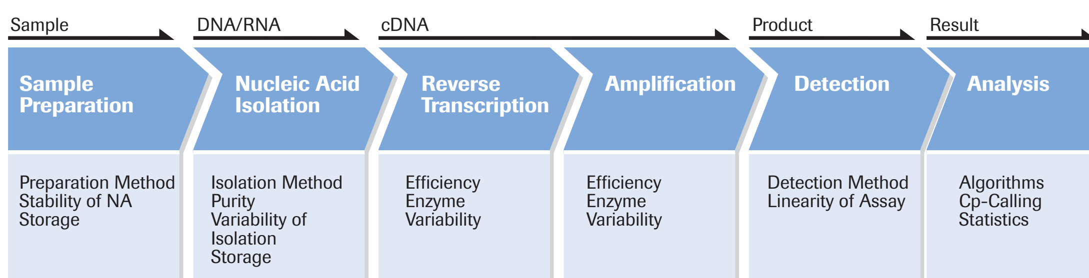


Figure 29: Criterias to be considered when planning a gene expression assay.

Starting Material

The quality and quantity of starting material is also crucial for optimal amplification. Always use highly purified template nucleic acid and a standardized extraction procedure throughout the entire study to minimize variations based on samples. For more information, see *Sample/Nucleic Acid Preparation*, page 62 in chapter 3, ***Assay Development: Tips and Tricks***

Controls

To make sure optimal reaction conditions are maintained during the experimental run, always include both negative and positive controls in the run. For details, see *Controls*, page 69 in chapter 3, ***Assay Development: Tips and Tricks***.

Kits and Reagents

Commercial master mixes, which are widely available for most of the Real-Time PCR platforms, can simplify assay optimization. Often, the master mix package insert lists the final working concentration of primers and probes and the cycling program settings.

For expression studies, RNA must be transcribed into cDNA. A one-step RT-PCR master mix reduces handling and analysis time. For two-step RT-PCR, reverse transcription enzymes or the Transcriptor High Fidelity cDNA Synthesis Kit are available.

Figure 30: LightCycler® 480 Probes Master, LightCycler® 480 SYBR Green I Master and LightCycler® 480 RNA Master Hydrolysis Probes

Alternatively, there are a number of commercially available assays containing either fluorescent dyes that bind directly to double-stranded DNA or fluorescently labeled, sequence-specific probes. Any of these can be used, provided the fluorophore carries fits the special requirements of the experiment and is compatible with the respective Real-Time PCR instrument. However, we highly recommend not using two detection formats on a single plate, as each is likely to require a unique channel setting or filter combination, as well as different cycling programs and/or acquisition modes.

2

Universal ProbeLibrary Assays

For maximum convenience and easy reaction set-up we recommend to use the Universal ProbeLibrary (UPL) Probes from Roche Applied Science:

With UPL, gene specific expression quantification assays are easily designed by using the web-based assay design center at www.universalprobelibrary.com.

A multiplex option allows to design an assay for the gene of interest together with one of the available pre-validated Universal ProbeLibrary reference gene assays.

The performance of an assay with a selected UPL probe follows established Real-Time PCR protocols using ready-to-use, hot-start masters designed for research studies. Depending on the Real-Time PCR platform you use, the following master mixes are available for use in combination with UPL probes:

- LightCycler® 480 System: LightCycler® 480 Probes Master
- LightCycler® 2.0 System: LightCycler® TaqMan® Master
- Other Real-Time PCR instruments: FastStart Universal Probe Master (ROX)

Figure 31: Universal ProbeLibrary Probes and website.

RealTime *ready* PCR Assays

Furthermore, Roche Applied Science offers RealTime *ready* PCR Assays. These pre-validated, ready-to-use expression assays are available as Focus Panels that target important classes of genes, or as custom panels and single assays (see page 71 for more information).

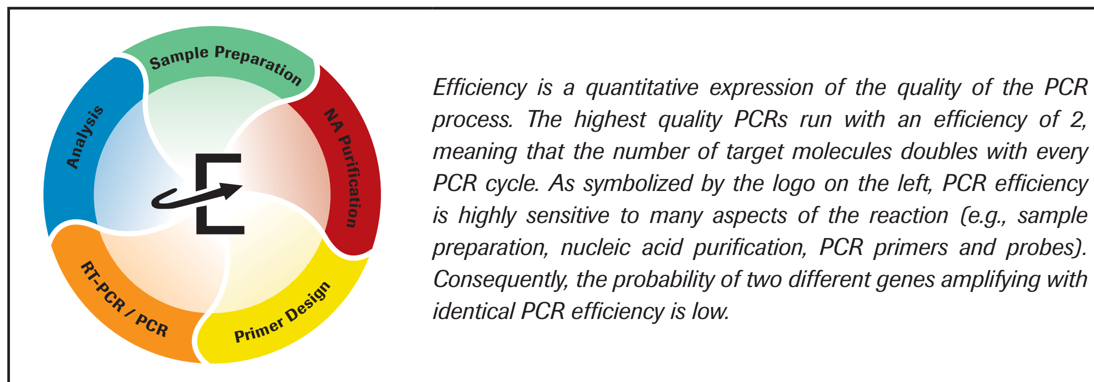
Assay Validation

The reliability of all quantitative Real-Time PCR applications and, consequently, of all relative quantification calculations depends on the quality of the PCR. By following the basic guidelines for PCR, one can design and optimize a quantitative assay. Nevertheless, PCR assays that require high sensitivity and accuracy should be validated with standard curves prior to use, as detailed below (see also *Evaluation of PCR Assays*, page 75 in chapter 3, ***Assay Development: Tips and Tricks***).

Efficiency

A characteristic marker for high quality assays is the PCR amplification efficiency. Evaluation of efficiency is essential for every Real-Time PCR gene quantification procedure.

To determine PCR efficiency for each target and reference gene, simply make serial dilutions of each template and determine the Cp value for each dilution. The resulting Cp values can be plotted versus the log concentration (or relative concentrations) of the template to generate a standard curve. By looking at how the ΔCp (distance from the Cp of one dilution to the next) varies with sample dilution, one can readily determine the quality of the PCR. Repeating standard curve runs can reveal much about assay stability.



Efficiency is a quantitative expression of the quality of the PCR process. The highest quality PCRs run with an efficiency of 2, meaning that the number of target molecules doubles with every PCR cycle. As symbolized by the logo on the left, PCR efficiency is highly sensitive to many aspects of the reaction (e.g., sample preparation, nucleic acid purification, PCR primers and probes). Consequently, the probability of two different genes amplifying with identical PCR efficiency is low.

Sensitivity

In order to determine assay sensitivity, a broad range of template dilutions should be covered. The regression line of the standard curve should reveal whether the assay yields consistent results over the range of initial template concentrations.

2

Precision

If replicates of each serial dilution are assayed, the precision of the standard curve can be determined. This will reflect the reproducibility and accuracy of the assay across the dilution range.

Poor efficiency or lack of consistency may be due to suboptimal reaction conditions, poor primer quality or purity, or suboptimal probe design.

In summary, data generated from a standard curve provides an excellent measure of the overall performance of an assay in terms of efficiency, precision, sensitivity, and stability.

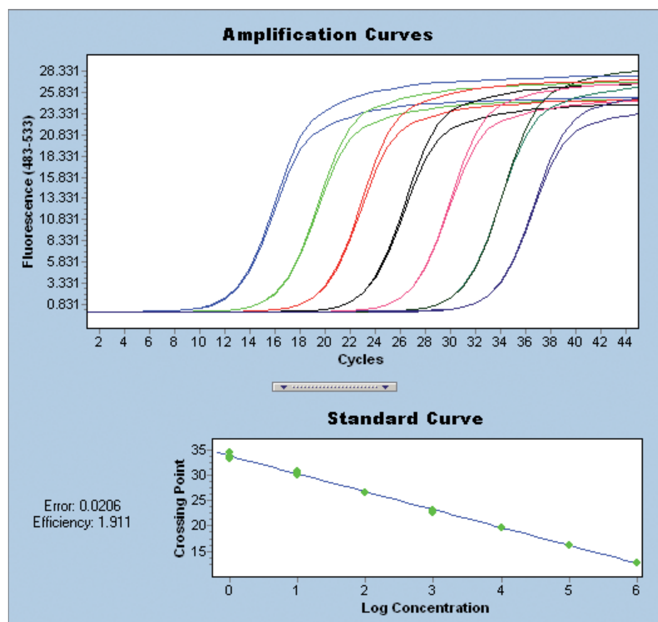


Figure 32: PCR efficiency derived from a standard curve. The slope of the standard curve gives a precise measure of the amplification efficiency of a target gene ($E = 10^{-1/slope}$). The expected slope for a 10-fold dilution series is -3.32 when the efficiency is 2, indicating that the template amount is doubling with each cycle. This efficiency value is automatically calculated for each standard curve and can be used to verify PCR quality.

Selecting an Analysis Method

Relative Quantification has expanded into a variety of applications. To provide sophisticated analysis options for all these applications, the LightCycler® System Software, especially the LightCycler® 480 Software, provides two different analysis modes for relative quantification.

The choice between the two analysis modes depends mainly on whether the amplification efficiencies of the examined genes are comparable or different:

The quick and easy **Basic Analysis**, which is ideally suited for optimized assays with comparable amplification efficiencies, offers a fully automated way to generate results with just one click.

The **Advanced Analysis** is based on standard curves. Here, the analysis includes the actual PCR efficiencies of all target and reference genes in the calculation of the final results. Therefore, this analysis guarantees a maximum degree of precision.

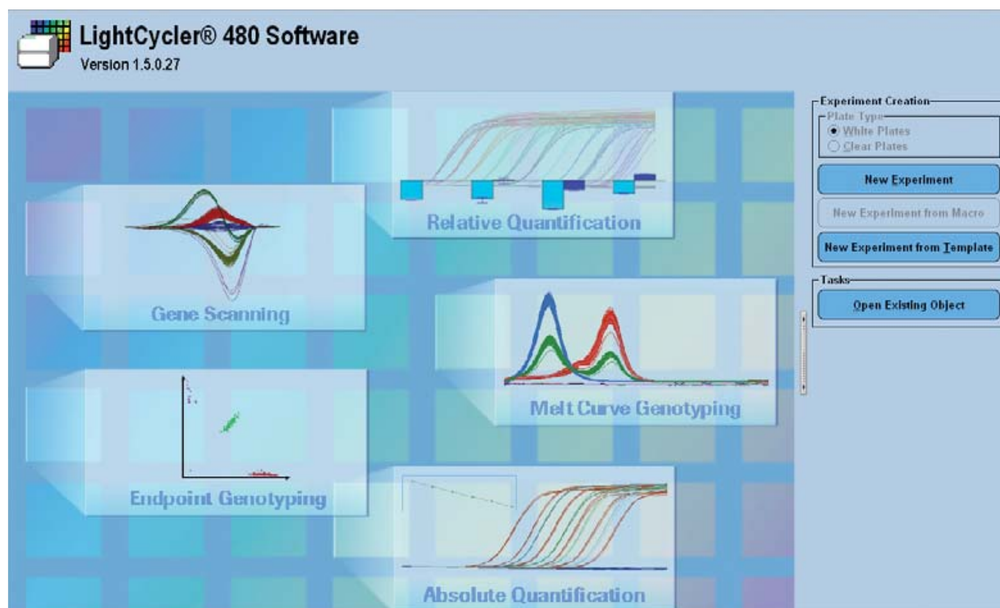


Figure 33: LightCycler® 480 Analysis Software.

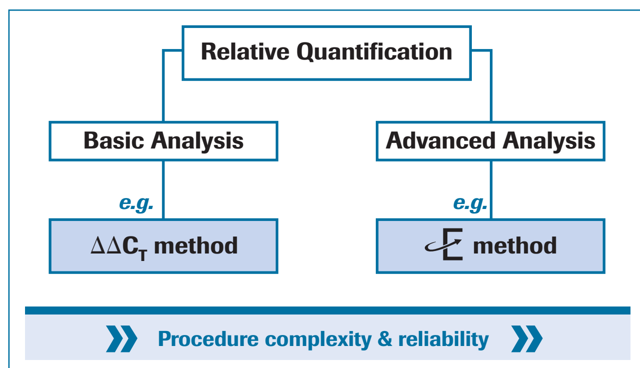


Figure 34: Relative quantification: Basic and Advanced Analysis methods.

2

	Basic Method (*e.g.* $\Delta\Delta C_T$ - Method)	**Advance Analysis (*e.g.* E - Method)**
Sample Types	Target (one / many) Reference (one / many)	Target (one / many) Reference (one / many)
Calibrator	Assay Calibrator and/or Study Calibrator[1]	Assay Calibrator and/or Study Calibrator
Standards (target/reference)	–	Standards
Efficiency	2 / ≠ 2 (linear)	2 / ≠ 2 (linear / non-linear)
Cp analysis	Fit Points method	▶ Second Derivate Maximum method ▶ Fit Points method

Figure 35: [1]**The LightCycler 480® Multiple Plate Analysis Software** provides an option to define a reference experiment (study calibrator), which can be used for normalization of other experiments.

Various mathematical models are established to analyze the relative changes in gene expression revealed by quantitative Real-Time PCR experiments.

The so-called **$\Delta\Delta C_T$ -Method** is a convenient and easy way to calculate the relative ratio as no standard curves are needed (Livak KJ & Schmittgen TD. *Methods* 2001 Dec; 25(4): 402-408).

The final, normalized relative ratio is calculated by the formula:

$$\text{normalized relative ratio} = 2^{-\Delta\Delta CT}$$

$$\Delta C_T = C_T\text{ (target) - } C_T\text{ (reference)}$$

$$\Delta\Delta C_T = \Delta C_T\text{ (sample) - }\Delta C_T\text{ (calibrator)}$$

Here an optimal doubling in both target and reference gene amplification is assumed (E=2). Therefore it is essential to verify this assumption by determining the PCR efficiencies of target and reference gene(s). In case the target and reference gene(s) do not have equal PCR efficiencies, you can either optimize or redesign the assay, or you can calculate the final result by using the PCR efficiency information (standard curve) as implemented in the Advanced Analysis.

The so-called E-Method provides an efficiency corrected calculation mode by using the determined PCR efficiencies of target and reference gene(s). The formula shown above implements additionally the efficiency of target (E_t) as well as the efficiency of reference (E_r):

$$\text{normalized relative ratio} = E_t^{\text{CT (target) calibrator - CT (target) sample}} / E_r^{\text{CT (reference) calibrator - CT (reference) sample}}$$

2

Basic Analysis

The Basic Relative Quantification module of the LightCycler® 480 Software permits straightforward analysis. This convenient module requires only a single mouse click to produce a result.

This module uses the $\Delta\Delta C_T$ Method to calculate Real-Time PCR results. This method provides a fast, simple, reliable way to generate relative quantitative results. You can use it when you are sure your assays are optimized and attain high PCR efficiency, or when highest accuracy of expression ratio values is not mandatory.

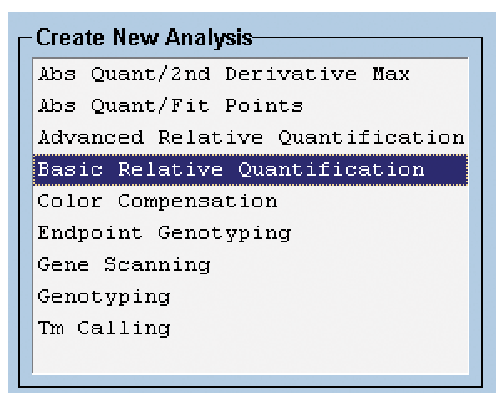


Figure 36: Selection menu for data analysis options in the LightCycler® 480 Software.

To use this method, create a new analysis and select "Basic Relative Quantification" from the menu that appears. The software immediately displays the final result of all unknown samples based on the $\Delta\Delta C_T$ method (which assumes E = 2.00 for both target and reference gene amplification).

For each sample, the result screen shows the target/reference ratio, as well the ratio normalized to a calibrator. These results are shown in three different ways: as a table, as a bar chart and as a sample view graph. They results can be summarized in a report or exported to another software program.

2

Figure 37: Options provided in the LightCycler® 480 Software for viewing relative quantification results.

By default, the Basic Relative Quantification module determines the Cp values automatically, using the so-called Fit Points mode. For this analysis, the reference gene(s) as well as the calibrator samples must be located on the same plate as the unknown samples (in-run).

If there is more than one reference gene on the plate, the software applies a pairing rule called “all to mean”. This rule pairs each target gene of interest with the mean value of all selected reference genes to generate the relative ratios shown in the final result.

To reflect actual experimental conditions, you can adjust the standard default settings of the $\Delta\Delta C_T$ method, including the following parameters:

- The efficiency of assays can be set to a previously determined actual efficiency value (*e.g.*, E = 1.87).
- The noise band can be adjusted to accommodate even non-uniform amplification curves.
- The display settings can be changed to show either mean or median results, as well as linear or logarithmic curves.

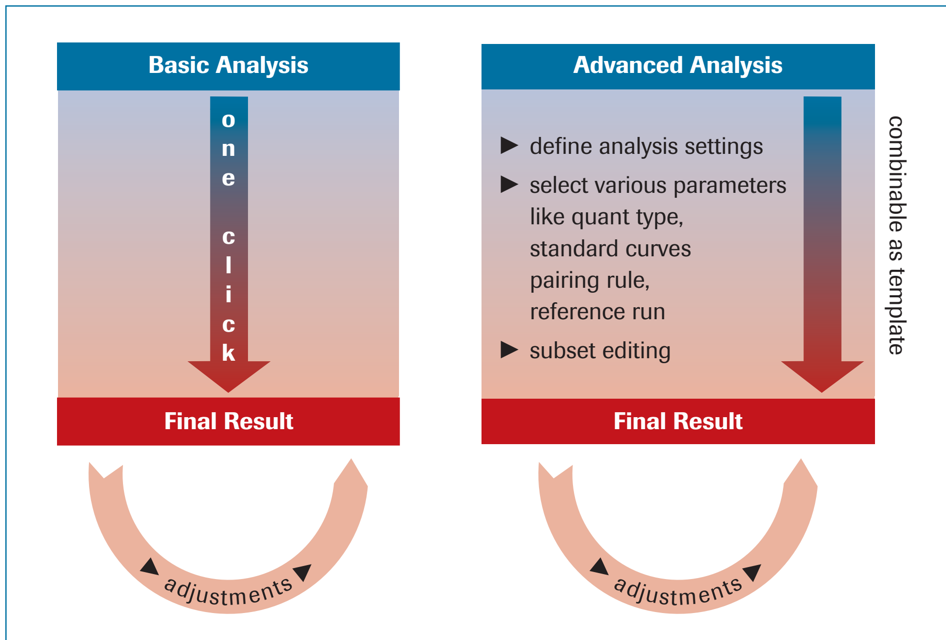


Figure 38

Advanced Analysis

If further refinement of results is desired or required, use the Advanced Analysis module, which can be highly customized. It uses additional algorithms to analyze even the most challenging research projects. It offers advanced users the flexibility to maximize data integrity. This method is recommended if you need to analyze complex data in an easy and straightforward way.

The pre-defined workflow of the E (Efficiency)–Method (below) provides an example of how to use advanced analysis to increase the reliability of results.

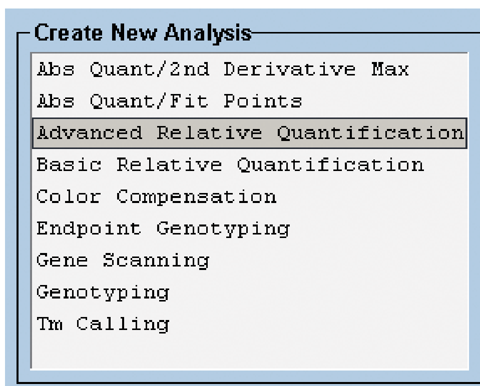


Figure 39: LightCycler® 480 Software 1.5 analysis methods.

E –Method

This method is recommended for all applications that need to track subtle changes in gene expression. It uses the information derived from standard curves to provide the highest possible assay precision for each reaction.

To generate standard curves, make serial dilutions of template, one for each target and one for each reference gene. Then, assay the serial dilutions and plot the resulting data. From this data, the software can calculate the actual PCR efficiency of each gene.

The Advanced Relative Quantification module of the LightCycler® 480 Software allows this standard curve data (for both target and reference genes) to be imported into subsequent experimental runs. It then uses actual PCR efficiencies to help calculate the relative gene expression ratios.

In most cases, standard curves for each target are generated when the corresponding assays are established and validated. Therefore, their data are readily available and can easily be imported. Alternatively, the diluted standards can be analyzed together with all unknown samples on the same plate.

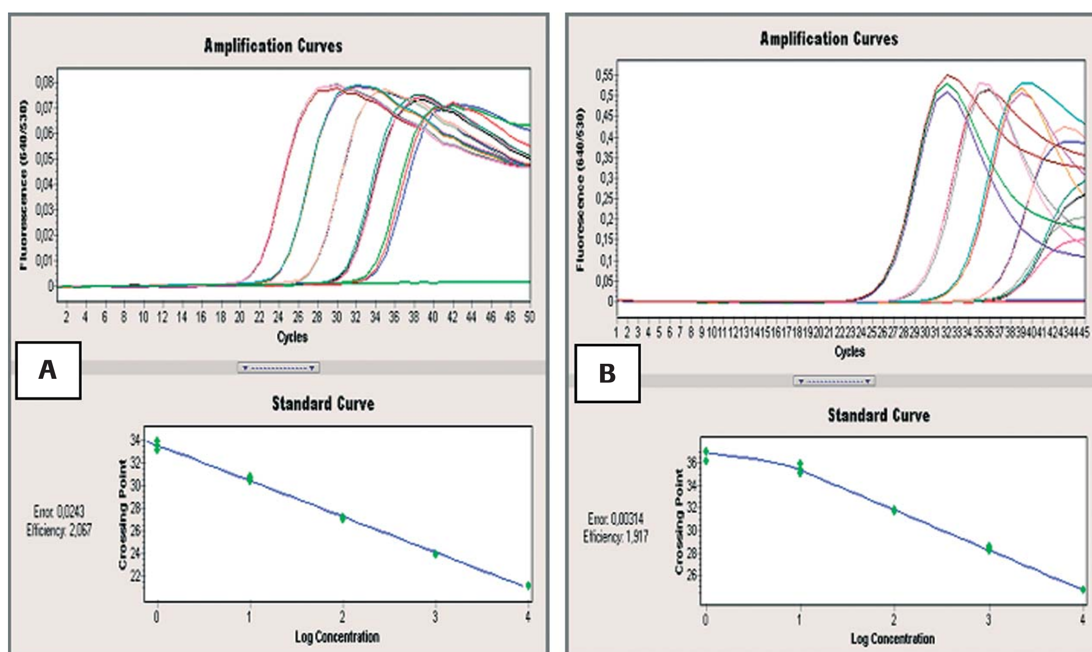


Figure 40: Establishment of standard curves for quantification at low concentrations.
Uniform data points always lead to a linear regression line. But if weak signals occur at low concentrations, the LightCycler® Analysis Software will automatically plot the "log concentration vs. Cp" as a non-linear curve that fits all the data instead of a straight (but erroneous) regression line.

Setting Options for the ⊰E –Method

Listed below are the parameters that need to be set for this method of relative quantification analysis.

The **2nd Derivative Maximum** method, for determining Cps, offers two different algorithms that handle data curves and especially background signals differently:

High Confidence: This algorithm is optimized for finding highly reliable Cps, *e.g.,* for data curves that have a sharp increase in fluorescence and, consequently, a high signal-to-noise ratio. This option drastically reduces the risk of false positive Cp calling. This version of the algorithm should be used for all experiments that require color compensation.

High Sensitivity: This algorithm detects Cps even for data curves that have a weak increase in fluorescence and a low signal-to-noise ratio. It is suitable for all assays that require very high sensitivity, *e.g.,* detection of low or single copy targets. The drawback of this algorithm is that it carries a certain risk of false positive Cp calling. The result of a High Sensitivity analysis should thus always be examined in detail.

Create new analysis
Abs Quant Type
Abs Quant/2nd Derivative Max
Sensitivity
High Sensitivity
High Confidence
Abs Quant/Fit Points
Surbordinate Abs Quant Analysis
Create by Target Name
- Create one analysis for each target name
Create by Filter Combination
- Create one analysis for each filter combination
Reference Analysis
Create In-Run
Select External
Pairing Rule
One To One
All To All
All To Mean
Mean To All
Default Standard Curve Settings
When there are no In-Run standards for a target name:
always use efficiency
allow external standards with matching target name

Figure 41

Each Relative Quantification analysis is based on two or more underlying absolute quantification analyses. To define how the software should identify the appropriate genes for a specific analysis, select one of the following options:

- **Create by Target Name:** Software identifies gene targets by the target name (assigned by user).
- **Create by Filter Combination:** Software identifies gene targets by the wavelength filter used to analyze it.

Reference data are usually created ***in-run***; this means target and reference gene assays are performed in the same run, using samples on the same plate. If a reference gene assay was run separately with samples on another plate (*e.g.*, because it required a different PCR program), use ***Select external*** to navigate to the database that contains the (previously run) reference data.

The different options under "Pairing Rules" provide tools for analyzing complex applications (*e.g.*, those involving multiple target and/or reference genes). **All To Mean** – the most commonly used option - analyzes data from each target gene against the mean value of all selected reference genes. **All To All** allows each target to be analyzed separately against each single reference gene. **One To One** pairs each target with only one reference gene, as defined by the (user entered) pipetting scheme. **Mean To All** calculates the mean value of all target genes and analyzes it separately against each reference gene.

2

The default standard curve setting - **”allow external standards with matching target names”** - simplifies import of external standard curves when no in-run standards are available. When this option is selected, the software automatically searches the database, then identifies and imports the standard curve file that has the same name as the target to be analyzed. Using real standard curves instead of manually edited efficiency values to calculate crossing points has the enormous benefit that unknown samples are evaluated only within the fixed dilution range covered by the standard curve, thus increasing the confidence level of the final result. If the Cp of a sample is located outside the dilution range of the corresponding standard curve, its final result will be marked in the “Status” column as “extrapolated standard curve”.

The Advanced Analysis method actually provides even more features and options (*e.g.*, settings, subsets, sample editing) that allow the user to customize the assay and analysis parameters.

The table below summarizes the analysis options for the Basic and Advanced Analysis methods:

	Basic	**Advanced**
Target Gene	one / many	one / many
Reference Gene	one / many	one / many
Pairing Target-to-Reference	All-to-mean	One-to-One All-to-mean All-to-All Mean-to-All
Calibrator	with / without	with / without
Assay Set-up on MWP	Target & Reference on same plate Full plate analyzed	Target & Reference on same plate Target & Reference on different plates Full plate, and/or Subsets analyzed
Cp Analysis	Fit Points Method	Fit Points Method 2nd Der. Max. Method
Efficiency	$E = 2 / \neq 2$	$E = 2 / \neq 2$ standard curves: linear or non-linear fit
Multiplex	monocolor / dual color	monocolor / dual color

Figure 43: Analysis options for basic and advanced quantitative analysis.

Analysis of Multiple Experiments

If several experiments, which were performed on separate microwell plates, need to be analyzed together, the analysis can be performed with the LightCycler® 480 Multiple Plate Analysis Software (for more information, see *Multiple Plate Analysis Software*, page 82 in chapter 3, ***Assay Development: Tips and Tricks***).

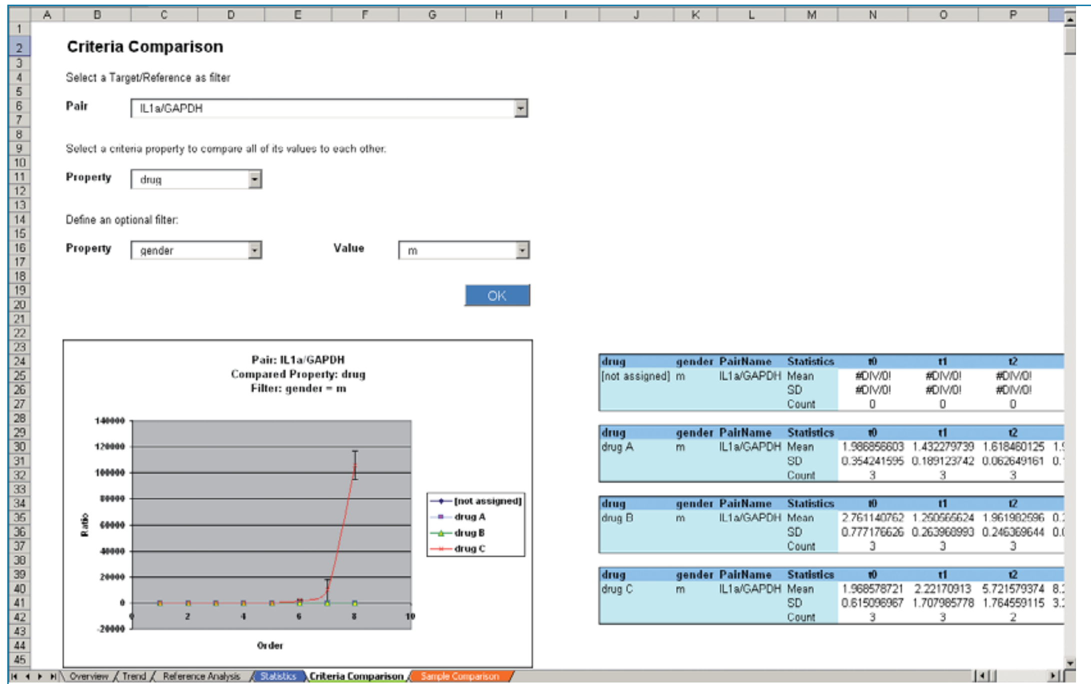


Figure 43: Analysis of multiple experiments using the LightCycler® 480 Multiple Plate Analysis Software.

2

Genetic Variation Analysis

Overview of Methods to Analyze Genetic Variations

Human beings show differences between their individual genomes on different levels: DNA sequences can carry single-nucleotide polymorphisms (SNPs), insertions, deletions, or variations in copy number of longer or shorter repeated elements. They can also vary in the way DNA is covalently modified, *e.g.*, by methlyation of CpG islands. Real-Time PCR offers different approaches to detect these different types of variation.

Single-nucleotide polymorphisms (SNPs) account for more than 90% of all genome sequence differences between individuals. In recent years, genotyping of SNPs has become a key component of genetic studies in fields like, *e.g.*, forensics, breeding of plants and animals, and especially pharmacogenomics (the study of how a specific human individium´s genotype is related to the way a person responds to a certain drug treatment). Identification and mapping of a dense set of SNP markers may make it possible to study the genetic basis of complex diseases via population approaches, or with the final aim to create personalized drugs that are individually tailored for each person's genetic make-up.

Figure 44: Single nucleotide polymorphisms.

In all genetic variation studies, a large number of individuals must be genotyped, in order to characterize a large number of markers. Alleles of known SNPs must be identified and called correctly, and the presence of newly arising variants must be detected. The ideal genotyping method must be robust, quickly developed without extensive optimization, easy to use, automatable, and scalable.

Before specific SNPs can be genotyped, whole genes or certain subfragments are often scanned to find out if previously unknown variations have arisen in a region of interest. While comprehensive sequencing would of course be the most straightforward method to do this, a PCR-based screening step allows reduction of sequencing sample number and cost because regions devoid of any unknown variations can be excluded from the sequencing runs.

With only a few exceptions, all current genotyping techniques require a PCR amplification step. In most techniques, the first step is PCR amplification of a desired SNP-containing region, to increase assay specificity and sensitivity.

Displaying amplification curves that cover the entire PCR process is not the only advantage provided by Real-Time PCR. When allele-specific hydrolysis probes are used, the signal generated by their cleavage will accumulate during amplification, and its endpoint value can be used to determine the genotype(s) present.

In addition to this most basic genotyping method, the LightCycler® System allows programming of so-called melting curves downstream of the cycling program. The data collected during this additional post-PCR step provide additional information about the melting behavior of the product, facilitating the identification of a specific SNP allele or allele combinations.

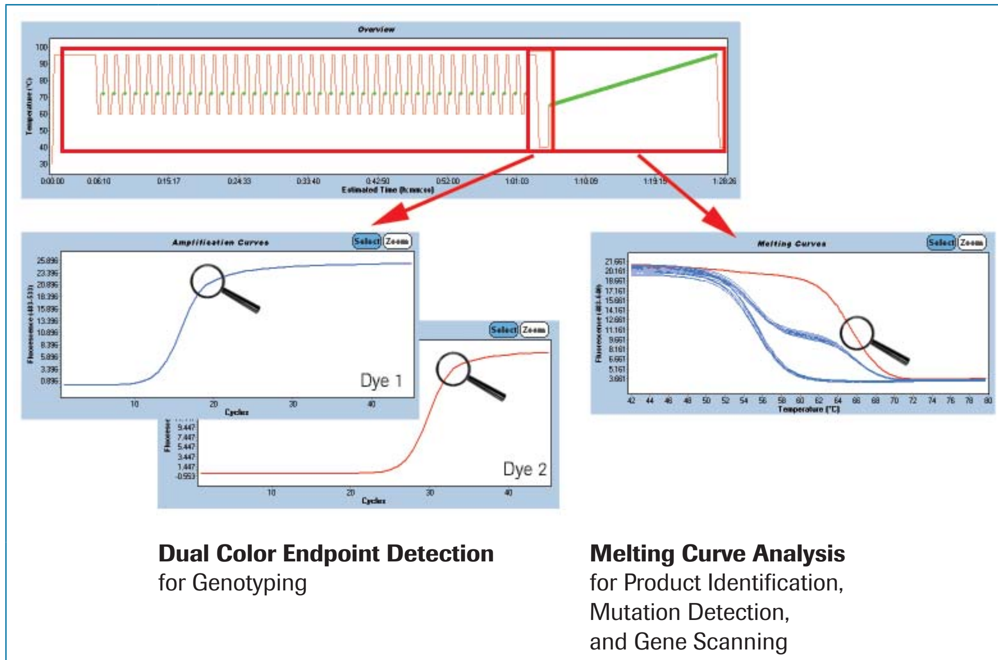


Figure 45: Different genotyping methods analyze fluorescent signals from different parts of a Real-Time PCR run.

2

Among current genotyping techniques, we can differentiate those that detect only known variants from those that detect any variants, including new ones.

For detection of **known variants**, two different methods of SNP analysis are available:

- The basic, most commonly used method is **Endpoint Genotyping** analysis using enzymatically cleaved hydrolysis probes.
- A more advanced method is **Melting Curve Genotyping** analysis using hybridizing HybProbe or SimpleProbe probes.

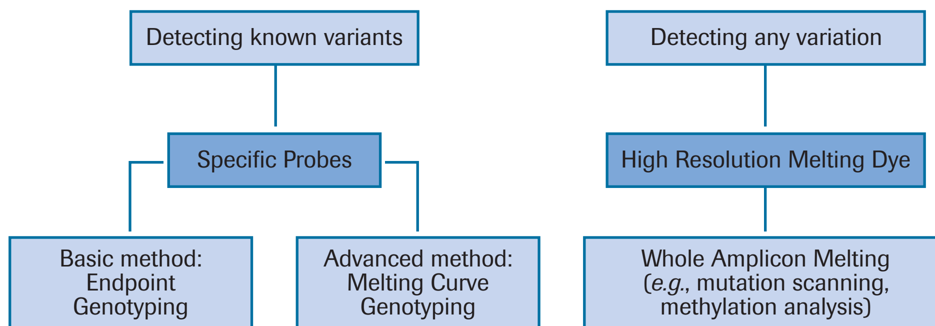


Figure 46: SNP analysis methods for the detection of known or unknown variants.

Gene Scanning is the discovery of **new variants** in target-gene derived amplicons. It can be easily carried out based on the analysis of the melting behaviour of these amplicons at high resolution in the presence of a saturating, DNA-binding dye. When combined with DNA bisulfite modification protocols, such high resolution melting approaches can also be adapted to the study of methylation patterns (see case study on page 126)

In more advanced setups, scanning for unknown and detection of known SNPs can even be combined in the same reaction: the combination of an unlabeled probe (covering the known SNP) and a saturating DNA-binding dye allows analysis of a defined polymorphism via **probe melting**, as well as a simultaneous scanning for further mutations via **amplicon melting**.

2

Detecting Unknown Variants

Gene Scanning by High Resolution Melting

High Resolution Melting (HRM) is a novel, post-PCR method that allows genomic researchers to screen for unknown mutations or modifications. It requires only PCR reagents, a simple primer pair for amplifying the gene of interest, a saturating DNA-binding dye, and an instrument with precise temperature control. The LightCycler® 480 System´s hardware, software and reagents have been designed to optimally meet these requirements.

Currently, the main application for the HRM method is Gene Scanning, *i.e.*, the discovery of new variations in target-gene-derived PCR amplicons.

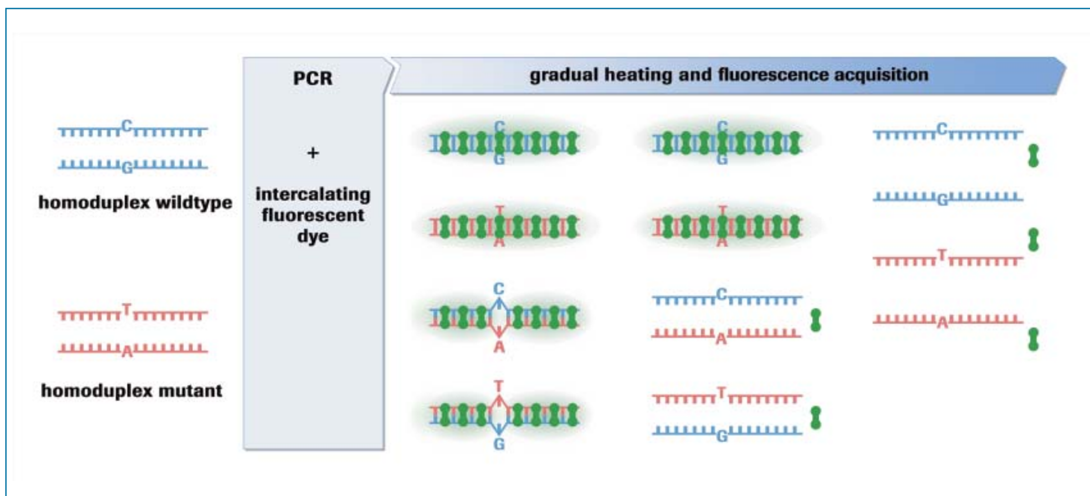


Figure 47: Melting of a heterozygote sample in the presence of a saturating DNA dye.
Note that both homo- and heteroduplex DNA molecules are present after PCR and contribute to the observed fluorescent signal.

Gene scanning information can be derived from a Melting Curve Analysis that is performed after PCR. Heterozygous DNA melts at a different temperature than homozygous DNA because of a sequence mismatch between hybridized strands. This difference in melting temperatures can be detected in the presence of a saturating, DNA-binding dye. The melting curve data, if displayed on a difference plot, will identify clusters of the samples (*e.g.*, homozygous wild type, homozygous mutant, and heterozygous).

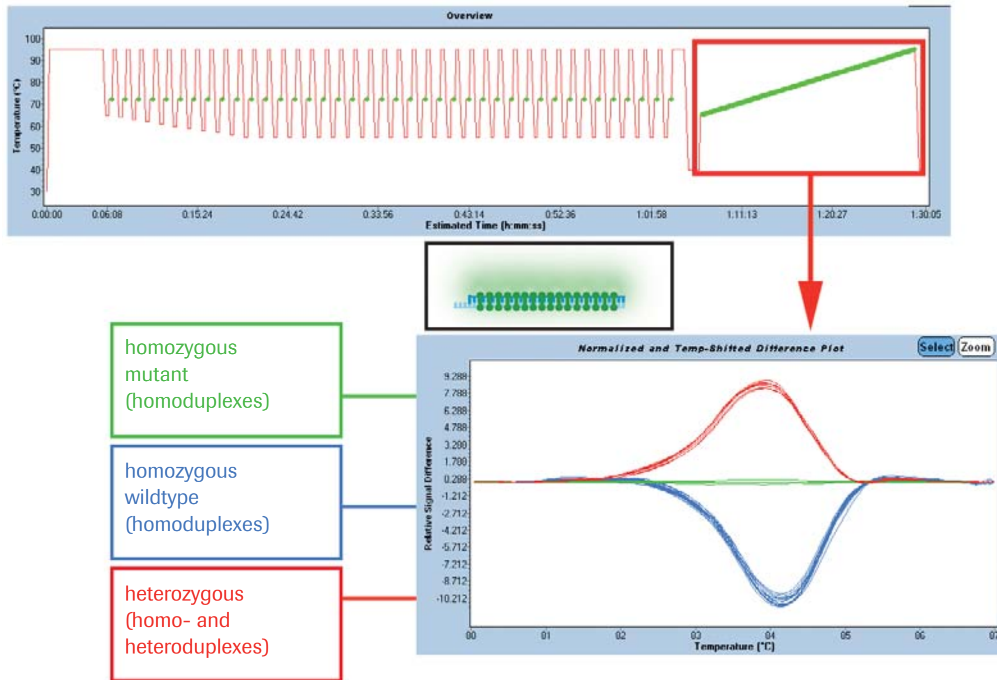


Figure 48: Difference plot analysis of fluorescence signals generated by post-PCR High Resolution Melting.

The Gene Scanning Software module available for the LightCycler® 480 Software normalizes the raw melting curve data to form a difference plot. The resulting difference plot clusters the sample into groups, based on the shape of their melting curves.

2

Optimizing HRM Assays

The main application of the HRM method is gene scanning, which allows you to screen for unknown mutations or modifications. However, to ensure an effective screening assay, the experiment must be designed carefully.

A single base variation affects the melting behavior of a 100 bp amplicon more than a 500 bp amplicon. Therefore, you should analyze amplicons no longer than about 250 bp. Longer amplicons can be analyzed successfully, but usually with lower resolution.

Amplicons contaminated with artifacts, such as primer dimers or nonspecific products, can make results difficult to interpret. Therefore, to obtain the best gene scanning results, design your primers carefully. Not all primers used for other assays before, or primers described in the literature, are necessarily suited for HRM analysis. Accurate primer design ensures specific primer binding at the desired target sequence and minimizes the formation of primer dimers. Primers should not bind at positions that have secondary structure. Always use highly purified (*e.g.,* HPLC purified) primers and low primer concentrations (*e.g.*, 200 nM each) to avoid primer dimer formation.

Since gene scanning analysis compares amplicons from independent PCRs, reaction-to-reaction variability must be minimized. One way to achieve this is to standardize the sample preparation procedure. Use the same amount of template in each reaction (5 to 30 ng template DNA in a 20 µl reaction). For optimal HRM analysis, all amplification curves should have a crossing point < 30. More importantly, check that all curves reach similar plateaus. Salts affect DNA melting behavior, so make sure that the concentrations of buffer, Mg^{2+} and other salts in the reaction are as uniform as possible for all samples.

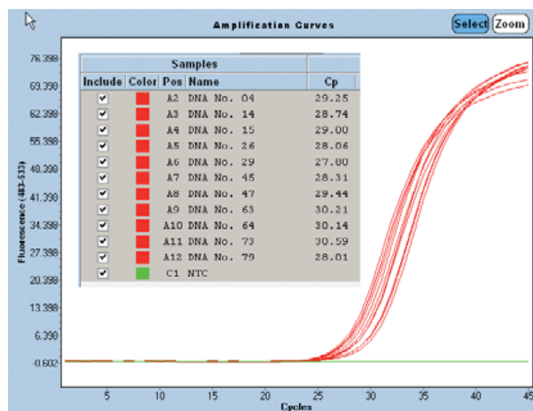


Figure 49: Samples included in one HRM analysis should show comparable PCR performance.

Allow sufficient data collection time for pre- and post-melt phases. Capture HRM data points within a range of approx. 10°C (or greater), centered around the observed melting temperature. This provides enough baseline data points for effective curve normalization and will result in better replicates and easier data interpretation.

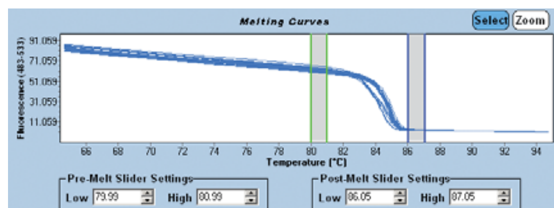


Figure 50: Correct setting of pre- and post-melt sliders is key for clear HRM results.

Note: For more information on optimizing and standardizing PCR sample preparation, see chapter 3, ***Assay Development: Tips and Tricks***, and the LightCycler® 480 Technical Note No. 1, ***High Resolution Melting Optimization Strategies*** available upon request.

Negative Controls

Negative, or no template controls (NTC) should be included in every HRM run.

Positive Controls

If available, controls that contain the wild-type and/or the mutant genotype, should be included in the run to verify the results.

Kits and Reagents for Genetic Variation Analysis

The convenient ready-to-use LightCycler® 480 master mixes are specially designed to support different detection formats used in gene scanning and SNP analysis.

For endpoint genotyping, use the LightCycler® 480 Probes Master, a ready-to-use reaction mix specifically developed for hydrolysis probe detection assays in multiwell plates.

The LightCycler® 480 Genotyping Master is designed for Real-Time PCR, followed by melting curve analysis on the LightCycler® 480 Instrument. It can be used to genotype single nucleotide polymorphisms (SNPs) and to analyze mutations. It is especially recommended for multiplex approaches. Please note that this master is not suitable for endpoint genotyping with hydrolysis probes, as it contains a 5'-3'-exo-minus Taq DNA Polymerase.

Consistent, high-quality gene scanning can be achieved with the LightCycler® 480 High Resolution Master that contains LightCycler® ResoLight, a novel dye that neither inhibits amplication enzymes nor interferes with PCR. This optimized master mix is highly stable and therefore is ideally suited for use in automated workflows.

Figure 51: LightCycler® 480 Probes Master, LightCycler® 480 Genotyping Master and LightCycler® 480 High Resolution Melting Master.

2

Other Applications of High Resolution Melting

There are numerous other possible applications for HRM (*e.g.,* mutation detection, DNA methylation analysis, species identification, etc.); labs worldwide are currently evaluating these applications.

A new method combines unlabeled probes (oligonucleotides which cover the SNP-containing region but do not carry a dye themselves) with a saturating, DNA-binding dye. Amplicons with sequence variations can be differentiated due to the shape of their melting curves.

In unlabeled probe melting, the melting curve differences occur over a very small temperature range, so the assay needs to have a higher resolution than other probe-based melting assays (*e.g.,* hybridization probe assays).

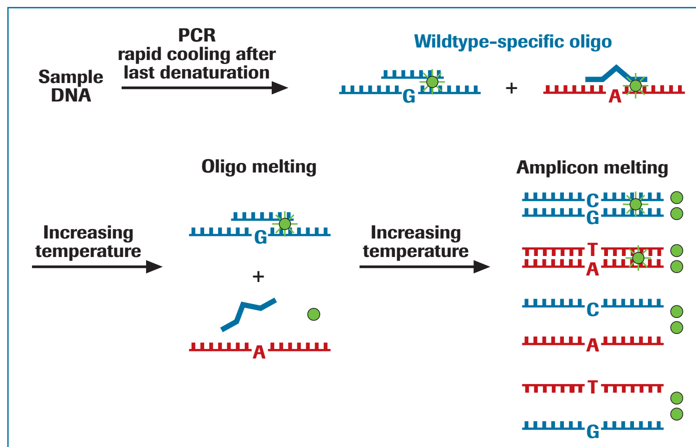


Figure 52: Principle of genotyping using an unlabeled probe in the presence of an HRM dye.

By combining probe melting (with unlabeled probe) and amplicon melting, this technique can simultaneously detect a specific SNP and scan the whole amplicon for other, new mutations.

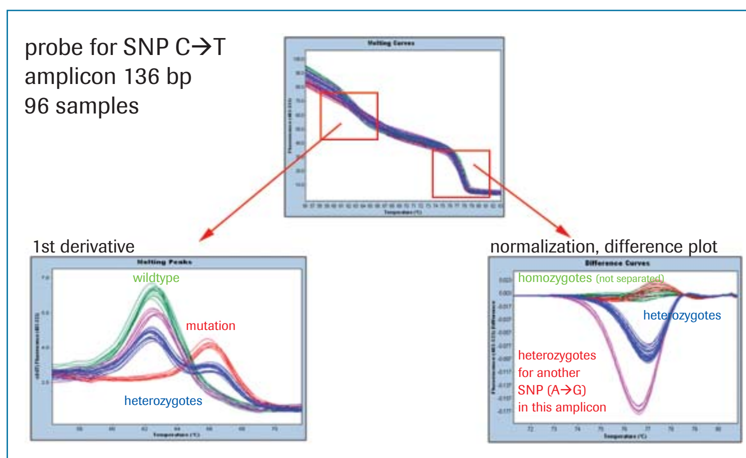


Figure 53: With HRM dyes, melting of unlabeled probes and whole amplicons can be studied in the same reaction at different temperatures.

2

Detecting Known Variants

Basic Method: Endpoint Genotyping

Endpoint Genotyping assays use hydrolysis probes (for an explanation of the working principle, see the *Detection Formats* chapter 1, page 18). Each probe contains two labels, a fluorescent reporter and a quencher, in close proximity to each other. Endpoint Genotyping analysis uses two sequence-specific probes that are designed to detect allele x and allele y and are labeled with different reporter dyes.

Standard set-up:

- FAM dye detects samples that are homozygous for allele X.
- VIC/HEX dye detects samples that are homozygous for allele Y.
- ➜ Endpoint genotyping is based on a dual color approach.

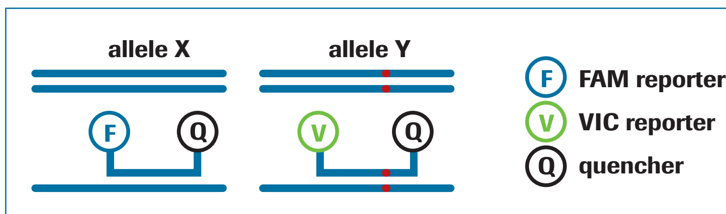


Figure 54: Dual-color principle of endpoint genotyping.

Data are collected throughout the PCR amplification. However, only the endpoint signal intensities of the two reporter dyes are used to identify the genotypes. The relative dye intensities can readily be visualized on a scatter plot, simplifying the discrimination of homozygous X, homozygous Y, and heterozygous samples.

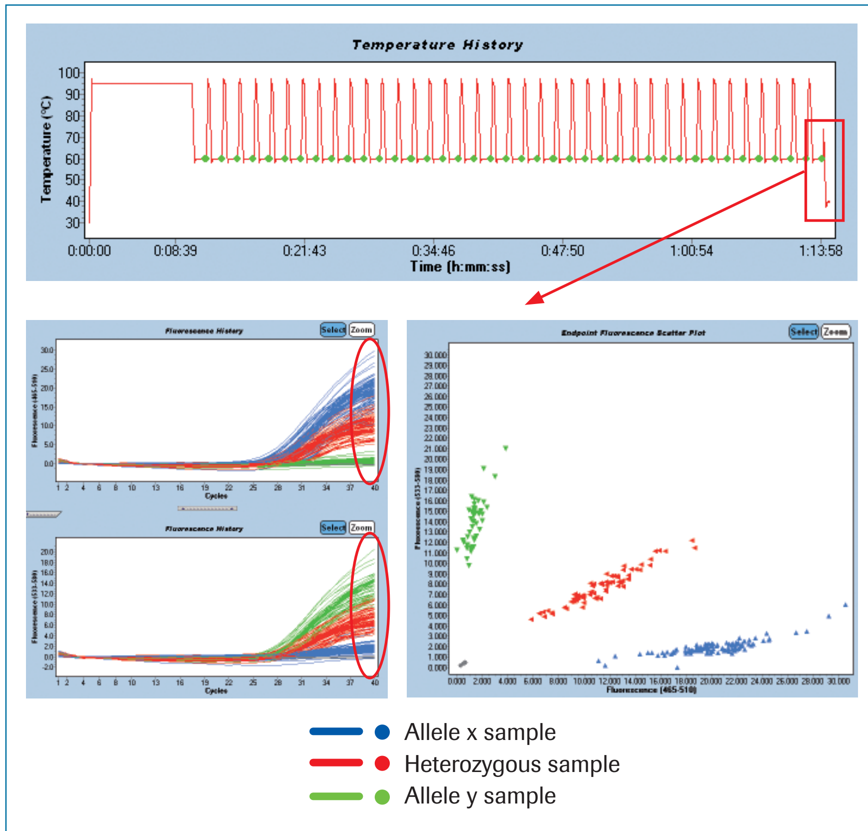


Figure 55: The Endpoint Genotyping module in the LightCycler® 480 Software groups samples with similar intensity distributions together and identifies each group as a genotype.

Pros and Cons of Endpoint Genotyping

Endpoint genotyping is a basic method that can be set up and performed quickly without optimization.

For many targets, ready-to-use primers and probes are commercially available and can be used under standard PCR conditions. This is a widely used, basic approach; the all-in-one reaction mixes are easy to use and results can be interpreted straightforwardly.

However, usually only one mutation per assay can be investigated, *i.e.*, the technique is not suitable for haplotyping (studying combinations of neighboring SNPs that are covered by the same probe). The method also generally fails in the presence of new, unexpected mutations.

Advanced Method: Melting Curve Genotyping

Melting curve analysis with HybProbe- or SimpleProbe probes is a reliable method for studying known variations (for more details about these probes, see *Detection Formats* on page 18). This method is highly accepted within the LightCycler® System community and many different assays based on it have been published. Allele-specific primers or probes are not needed; the same probe sequence is used for all alleles of an investigated SNP.

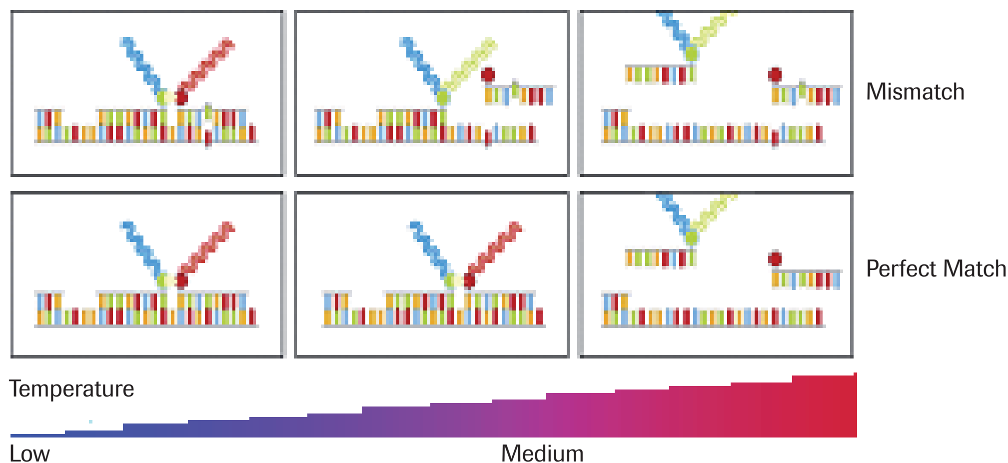


Figure 56: Principle of melting curve genotyping.

As the sequence-specific achor and sensor probes bind next to each other on the target DNA, fluorescence signal is generated via the FRET process. Since the temperature increases during melting curve analysis, the fluorescence signal decreases as the probes melt away from the template DNA. A single base change occuring within the region recognized by the probe will lead to thermal destabilization of the probe-target complex. Thus, the melting temperatures (T_ms) will be different for amplicons with sequence differences (SNP alleles). Therefore, genotyping information can be derived from the shape of a melting curve.

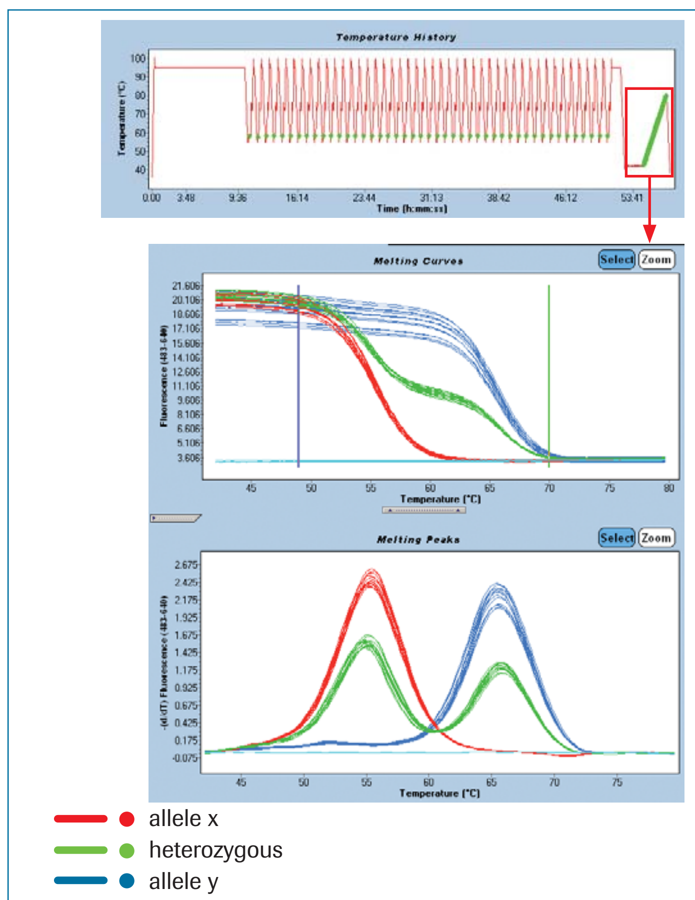


Figure 57: The Melting Curve Genotyping module in the LightCycler® 480 Software groups samples with similar melting profiles together and identifies each group as a genotype.

Pros and Cons of Melting Curve Genotyping

Highly flexible melting curve genotyping provides more insights into complex genetic constellations than basic hydrolysis probe chemistry. It even allows analysis of several variable sites in combination (*e.g.*, haplotypes). When used in multiplex assays, this technique can detect multiple SNPs. The superiority of this method to detect new mutations has also been documented (see Teupser, D., W. Rupprecht, *et al.* (2001). “Fluorescence-based detection of the CETP TaqIB polymorphism: false positives with the TaqMan®-based exonuclease assay attributable to a previously unknown gene variant.” *Clin Chem* **47**(5): 852-7.).

On the other hand, melting curve analysis requires careful design to make sure that the probe sequence covers at least one SNP, and optimization of each assay.

2

LightCycler® 480 Software for Genetic Variation Analysis

To speed up the process of setting up an experiment, the LightCycler® 480 Software provides run templates for many applications.

In addition, the LightCycler® 480 Software, Version 1.5 supports all the different analysis modes for finding genetic variations:

- Endpoint Genotyping
- Melting Curve Genotyping
- Gene Scanning

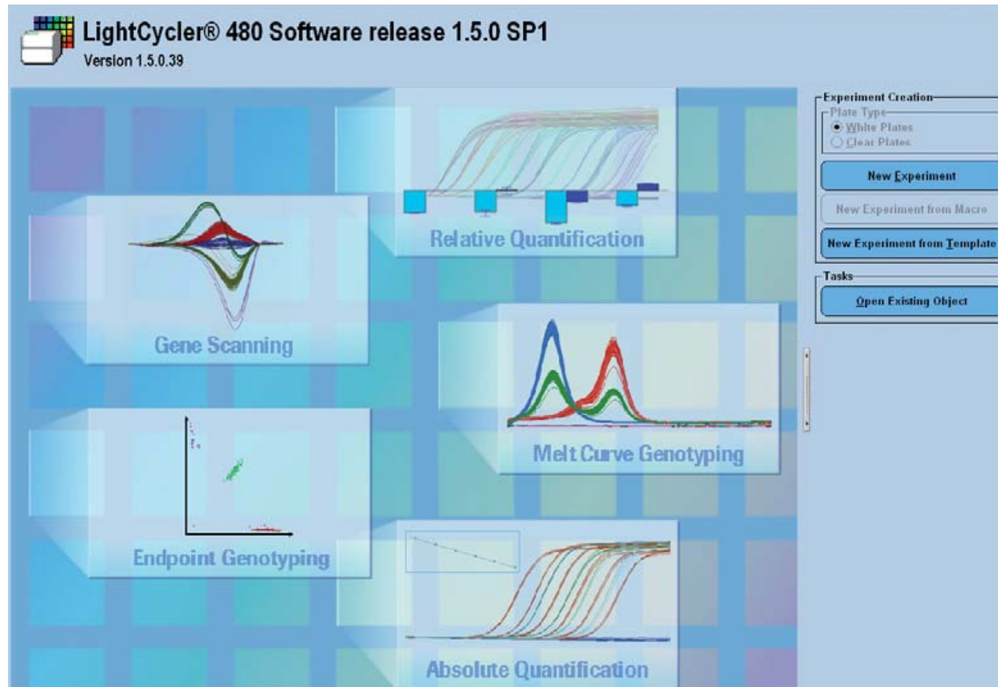


Figure 58: The LightCycler® 480 Software, Version 1.5 provides different fully automated analysis modules for finding genetic variations.

2

LightCycler® 480 Multiple Plate Analysis Software

To facilitate analysis of data generated from comprehensive gene expression or genotyping studies performed on the LightCycler® 480 System, Roche Applied Science offers an optional software module, the LightCycler® 480 Multiple Plate Analysis Software. This module can combine and compare analysis results previously generated by other modules of LightCycler® 480 Software, Version 1.5. It can also create studies that summarize the results from multiple experiments. For more information, see *Multiple Plate Analysis Software*, page 82 in chapter 3, ***Assay Development: Tips and Tricks***.

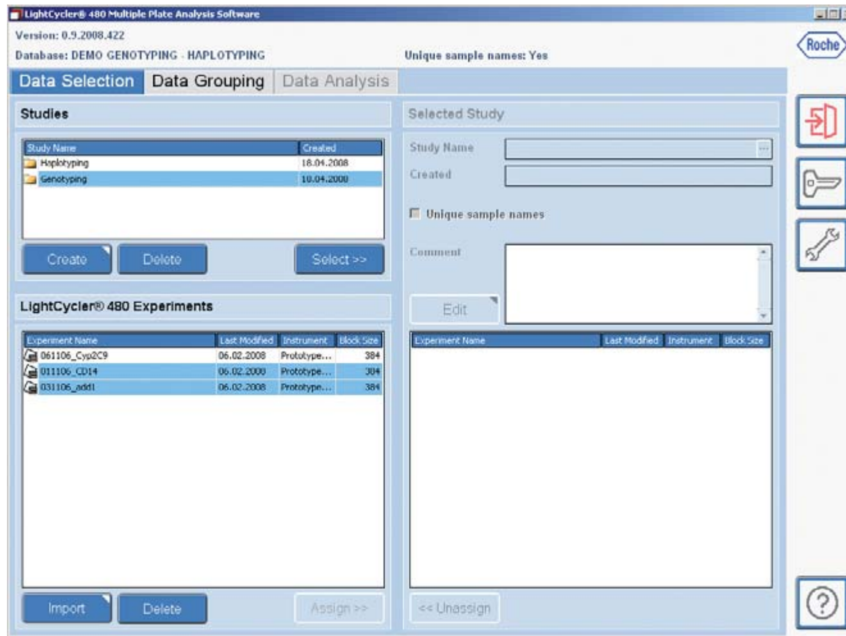


Figure 59: LightCycler® 480 Multiple Plate Analysis Software.

2

3

Assay Development

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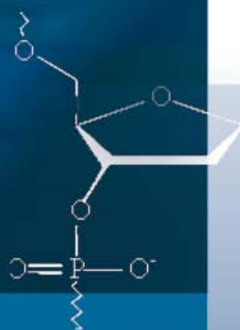

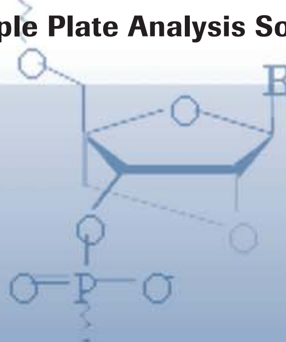

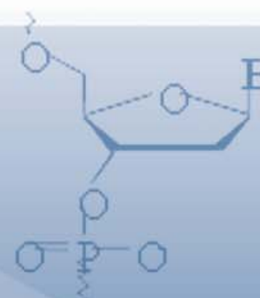

3

Tips and Tricks

PCR Requirements

The hallmarks of valuable qPCR or genomic variation studies are accuracy and applicability. These two properties in turn, depend upon several critical factors:

- High quality template DNA/RNA
- Specific and compatible PCR primers and probes
- Highly efficient and robust PCR
- Sensitive and precise detection

Therefore, getting accurate and reproducible results requires careful planning of the PCR assay and adapting it to experimental requirements. As with any experimental approach, the initial time spent on optimizing the PCR will pay off in the long run.

Sample/Nucleic Acid Preparation

The most important determinant of reproducibility and biological relevance of any PCR assay is undoubtedly the quality of the template nucleic acid (NA). Many samples are unique and irretrievable. Consequently, some effort should be made to standardize the entire purification process, from collecting, transporting, and storing samples to extracting and purifying nucleic acids from them.

To obtain DNA or RNA that is pure, intact and free of PCR inhibitors, we recommend the use of the following Roche Applied Science products:

- High Pure Kits for manual nucleic acid isolation
- MagNA Lyser and MagNA Pure Instruments (described below) for automated nucleic acid isolation

The **MagNA Lyser Instrument** is used to liquefy solid sample materials and to efficiently homogenize mammalian or plant tissue.

The **MagNA Pure Compact System** can automatically process 1-8 samples in 22 to 40 minutes. This small instrument uses pre-filled, ready-to-use reagents and disposable tubes to set up the run quickly, conveniently, and safely. The operating software has a touch-screen interface that is easy to navigate. The software allows you to select one of the pre-installed protocols by simply scanning the barcode on the reagent cartridges. The instrument simplifies experiment documentation with its state-of-the-art user management system and its ability to export data into an external database via host connectivity.

The **MagNA Pure LC Instrument** is a robotic workstation that can automatically process up to 32 samples. This flexible purification system can prepare almost any nucleic acid from almost any sample with the help of appropriate kits. The instrument is easy to use and has streamlined, easily navigable software.

Figure 60: MagNA Lyser, MagNA Pure Compact and MagNA Lure LC 2.0 instruments.

In addition to serving as a workstation for automatic nucleic acid preparation, the MagNA Pure LC system can also be used as a pipetting robot for setting up PCR samples and speeding up your routine reaction preparation procedures. There are several available MagNA Pure LC Cooling Blocks that support these so-called post-elution functions, *e.g.*, the addition of purified nucleic acids to PCR mixes suitable for LightCycler® System analysis.

Store isolated nucleic acids at high concentrations and in aliquots. Avoid repeated freezing/thawing of these aliquots. If the nucleic acid concentration is low, add carrier NA (*e.g.*, MS2 RNA, 10 ng/µl) to the samples, and store them in siliconized tubes.

The MagNA Pure LC System includes kits and purification protocols optimized for the preparation of DNA, RNA or total nucleic acids from many different sample materials, *e.g.,* blood, cells, body fluids, bacterial cultures, paraffin-embedded tissue, or plant material. The instrument generates a high quality nucleic acid, suitable for use in PCR on the LightCycler® Instruments.

Primer Design

Accurate primer design ensures that the primers bind specifically at the desired target sequence, do not bind at positions that have secondary structure, and do not generate primer dimers.

The most important region in the primer is the 3´ region, because amplification starts here. In general, these ends should be free of secondary structure, repetitive sequences, palindromes, and highly degenerate sequences. Forward and reverse primers sequences should not be complementary to each other, especially at their 3´ ends; they should have equal GC content, ideally between 40% and 70% GC. Whenever possible, avoid an unbalanced distribution of G/C and A/T-rich domains and make primers 16-24 bases long.

Always use highly purified primers (*e.g.*, HPLC), since shorter primer fragments (by-products of manufacturing) enhance the formation of primer dimers.

For selection of primers commercial software is available, *e.g.*, Primer3 (http://frodo.wi.mit.edu/primer3/input.htm) or **LightCycler® Probe Design Software 2.0**, which can be used not only for selection of probes but also for identification of primers.

BLAST the primer sequences to ensure they are specific for the target species and gene (http://www.ncbi.nlm.nih.gov/BLAST).

Design PCR primers that have annealing temperatures around 55 - 60°C and use normal primer concentrations (*e.g.*, 500 nM each).

In general, amplicons up to 1000 bp long are possible, if reaction times are suitably extended. However, shorter amplicons (< 300 bp) are optimal for most Real-Time PCRs.

Hydrolysis probe-based assays require that amplicons be kept short (< 150 bp). High Resolution Melting applications work best with < 250 bp amplicons.

Use optimal primers (and probes) for highly specific PCR assays. Rely on LightCycler® Probe Design Software 2.0 (Cat. No. 04 342 054 001) to design your primer and probes, even for the most demanding multiplex assays.

3

Probe Design

Recommendations for Hydrolysis Probes

In silico, ready-to-use hydrolysis probes are commercially available for a wide range of target genes. The protocols provided for them generally do not require further optimization but may not always lead to the expected results. Therefore, the following more flexible solutions should also be considered.

Universal ProbeLibrary (UPL)

For maximum convenience and easy reaction design, we recommend using Universal ProbeLibrary (UPL) probes from Roche Applied Science. You can design gene expression assays and quantify virtually any transcript in any genome by using the unique combination of the 165 pre-validated UPL probes and rapid, online assay design using the ProbeFinder software tool freely accessible on

www.universalprobelibrary.com

The Universal ProbeLibrary (UPL) allows you to design real-time qPCR assays with just a few clicks and to analyze over five million transcripts from a variety of organisms. When a transcript sequence or sequence ID number for a gene of interest (GOI) is entered, the ProbeFinder software displays a list of primer-probe combinations that can be used to generate an assay for that transcript.

Adresse https://www.roche-applied-science.com/servlet/RCConfigureUser?URL=StoreFramesetView&storeId=10201&catalogId=10201&langId=-3&countryId=de

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Home > Special Interest Sites > Genomic Systems > Real-Time PCR Systems > **Universal ProbeLibrary System**

Universal ProbeLibrary

- Real-Time PCR Systems
  - LightCycler® Carousel-Based System
  - LightCycler® 480 System
  - **Universal ProbeLibrary System**
    - System Description
    - Technology
    - Assay Design Center
    - User Statements and Application
    - Assay List
    - Performance
    - Product List
    - Support
    - Literature and References
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Gene Expression Quantification with Real-Time PCR - Simple and Fast

- Design real-time qPCR assays online in seconds.
- Rely on just 165 prevalidated probes for over five million qPCR assays for a large variety of organisms.
- Reduce the cost of gene expression analysis by performing multiplex qPCR assays with Universal ProbeLibrary Reference Gene Assays.
- Perform real-time PCR on any real-time PCR instrument with standard protocols.

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View Universal ProbeLibrary Multi Media Presentation

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Figure 61

The short UPL hydrolysis probes are labeled at the 5´ end with fluorescein (FAM) and at the 3´ end with a quencher molecule.

Each UPL probe can recognize several targets because probe sequence are very short (8-9 nucleotides) and carefully selected based on a statistical analysis of various organisms´ transcriptomes. Structurally based on *Locked Nucleic Acids (LNAs)*, they hybridize to their target sequence much more strongly than standard DNAs. Thus, when combined with target-specific primers, they can be used as components of highly specific assays.

UPL assays are compatible with established Real-Time PCR protocols and all Real-Time PCR instruments that can detect fluorescein, FAM, and/or SYBR Green I. UPL probes may be purchased either individually or as part of preconfigured sets of 90 probes each (available for human, mouse and rat genomes).

UPL probes can be combined with Universal ProbeLibrary Reference Gene Assays in a dual-color setup, allowing you to easily quantify the expression levels of a human, mouse, or rat gene relative to the expression levels of an endogeneous reference gene. All other required reaction components are available as components in ready-to-use hot start PCR master mixes.

Use Universal ProbeLibrary probe: #64, cat.no. 04688635001

Primer	Length	Position	Tm	%GC	Sequence
Left Primer	18	382 - 399	60	56	ccaaccgcgagaagatga
Right Primer	20	459 - 478	59	60	ccagaggcgtacagggatag

Amplicon (97 nt)

ccaaccgcgagaagatgacccagatcatgtttgagaccttcaacaccccagccatgtacg
ttgctatccaggctgtgctatccctgtacgcctctgg

Download pack insert | PDF report | Text report | Order probes or set

Run in multiplex PCR with

HPRT

64
1
1761

ccaggctg ga
cccagatcatgtttgagaccttcaacaccccagccatgtacgttgctatccaggctgtgct
400 438 460

Figure 62: Result of an assay design (selected UPL probe and recommended primers) as provided by the web-based Roche Applied Science Assay Design Center.

- **Significantly reduce assay design time** – in just seconds, design specific, intron-spanning assays for multiple organisms with the free, web-based ProbeFinder software, available at www.universalprobelibrary.com.
- **Rely on probe-based qPCR assays that work the first time** – there is no need to wait for custom probe synthesis or spend weeks on assay optimization.
- **Reduce the costs of gene expression quantitation** – by performing multiplex assays with UPL Reference Gene Assays.
- **Utilize standard protocols on any Real-Time PCR instrument** – no special hardware or reaction conditions required.

3

Recommendations for HybProbe Probes

The hybridization probe format uses two specifically designed oligonucleotides that hybridize, side by side, to an internal sequence of the amplicon during the annealing phase of PCR. One probe is labeled at the 3´ end with fluorescein; the second probe is labeled at the 5´ end with LightCycler® Red and phosphorylated at its 3´ end. These two dyes are close to each other (gap of 1 - 5 bases between the probes) only when the two oligonucleotides anneal to adjacent regions on the target, making fluorescence resonance energy transfer (FRET) possible.

When designing probes, avoid sequences that can hybridize to the 3´ termini of PCR primers, and that are repetitive, monotonous or self-complementary (for easy design of hybridization probes, use LightCycler® Probe Design Software 2.0.)

LightCycler® Probe Design Software 2.0 supports design for different experiment types
- ▶ Primer/probe sets for quantitative PCR
- ▶ Primer/probe sets for mutation detection
- ▶ Primers for PCR using the SYBR Green I detection format

Multiplexing capability
- ▶ up to 4 different HybProbe pairs (LightCycler® Red dyes/Fluorescein)

Supports different detection formats
- ▶ HybProbe probes
- ▶ SYBR Green I (primers only)
- ▶ SimpleProbe probe (for mutation detection)

Reaction conditions
- ▶ Roche generic buffers can be used

Designed primers can be directly submitted for BLAST search

For mutation detection, one probe, the *anchor* probe, hybridizes to a part of the target sequence that is not mutated. The second probe, called the *sensor* probe, covers the predicted site of the mutation. The *sensor* probe´s melting temperature (T_m) should be at least 4 °C lower than the *anchor* probe´s, and 5-10 °C higher than the primers´ T_m.

A mismatch between the target sequence and the *sensor* probe may have a greater or lesser effect on the probe-target hybrid, depending on the nature of the mismatch. The stability of different mismatches sequences in a hybrid is given below:

most stable — **least stable**

G:C > A:T > G:G > G:T = G:A > T:T = A:A > T:C > A:C > C:C

If the mutation probe binds to the strand that produces the least stable mismatch, it will produce a higher ΔT_m. The ΔT_m between a perfect match and mismatch should be at least 4 - 5 °C.

For maximum convenience, use companies licensed by Roche Applied Science to synthesize the probes:

Company (Headquarter)	Area of Resposibility	Contact	Design
TIB Molbiol (Germany)	worldwide, w/o Japan	www.tib-molbiol.com	×
Proligo (France) now Sigma/Aldrich	worldwide	www.prologio.com	×
Metabion (Germany)	Europe	www.metabion.com	×
Idaho Technology (USA)	USA	www.idaho.com/itbiochem	×
NGRL (Japan)	Japan	www.ngrl.co.jp	×

Recommendations for Multiplex Assays

More complex PCRs require simultaneous detection of several targets with different colored probes (multicolor detection). Multiple target sites can be located either on one amplicon (monoplex) or on several amplicons (multiplex). For multiplex assays, there are always multiple primer sets in the reaction. The simplest multiplex PCR assay involves one target gene detected in one channel while a control gene is detected in another channel (duplex PCR with internal control).

For optimal multiplex results, select product lengths of ≤ 350 bp. Amplicons should not be larger than 400 bp.

When setting up multiplex assays, first establish and optimize the individual monoplex reactions. Be aware that all assays must work under the same PCR conditions. Perform each combination of assays, *e.g.* for quadruplex PCR, perform monoplex, duplex, triplex, and then quadruplex.

Use the same concentration/copy number for each template.

For quantification: Check the dynamic range of your multiplex assay by diluting one template while keeping the concentration of the others constant.

For melting curve analysis: If additional melting peaks are visible, you can usually improve performance by using asymmetric PCR, *i.e.* favoring the amplification of the DNA strand to which the hybridization probe probes bind.

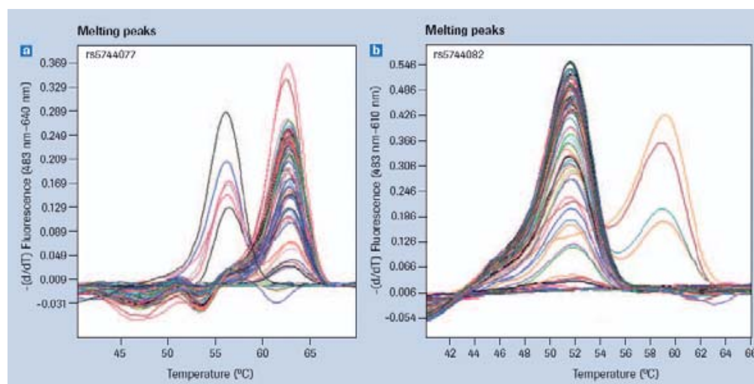


Figure 63: A typically melting curve analysis is shown for two different SNPs.

Recommendations for SimpleProbe Probes.

To design a successful SimpleProbe probe, pay particular attention to the reverse complementary region (DNA region that the probe binds). Use an artificial reverse complementary oligo to test probe function. The interior of the SimpleProbe probe should always cover the mutation site; the sequence covering the mutation should not be too close to the 3´or 5´ ends of the probe. The region of the target that is directly below the fluorescence label (positions -1 and -2) should contain C or T; having a G at position -1 or -2 should be avoided.

To create a stable alignment between the reporter dye and the double-stranded DNA, do not allow wobble bases at binding positions 1 – 4. In some cases, secondary structure may negatively affect the function of single-labeled probes. In such cases, we recommend using hybridization probes rather than SimpleProbe probes.

For rapid design of SimpleProbe probes, use the LightCycler® Probe Design Software 2.0 (Cat.No. 04 342 054 001).

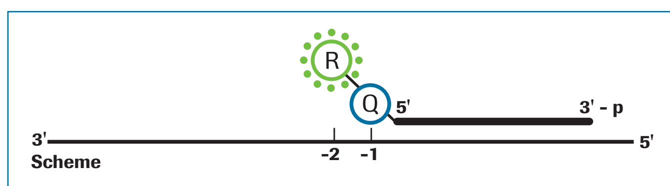


Figure 64: Design of SimpleProbe Probes.

3

Recommendations for Unlabeled Probes

In order to avoid elongation during PCR, an unlabeled probe must be 3´-phosphorylated. The probe should be relatively long (25-33 bases), so it can bind enough dye for a detectable signal. Preferably, it should have a high GC content; 55-70% GC works best. The SNP should be at least 3 bases away from the ends of the probe.

The template must be amplified by asymmetric PCR to produce an excess of the probe-binding strand.

e.g., final conc./rxn: 0.1 µM forward primer
0.5 µM reverse primer
0.5 µM unlabeled probe

If the signal generated by probe melting is too low, it can sometimes be improved by:

- Using a longer probe
- Shifting the probe recognition sequence 2 or 3 bases up- or downstream

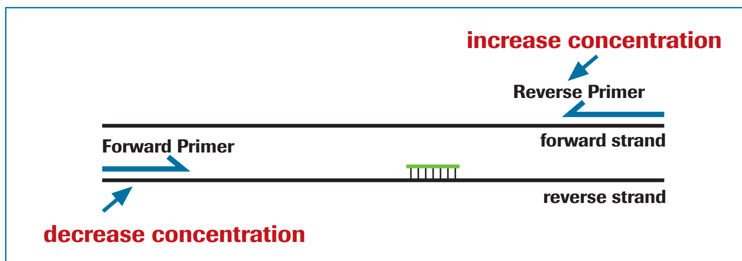


Figure 65: Asymmetric PCR uses unequal amounts of primers to favor synthesis of the strand (here: reverse strand) to which the detection probe can bind.

Optimizing Real-Time PCR

Good laboratory practice is an important prerequisite for successful Real-Time PCR. Specifically, always optimize the entire experimental workflow to minimize the risk of cross-contamination between samples and the chance of carrying nucleic acids over from one experiment to the other.

Note: the *Roche Applied Science PCR Applications Manual, 3rd edition*, is available for more background information on general – including non-real-time PCR.

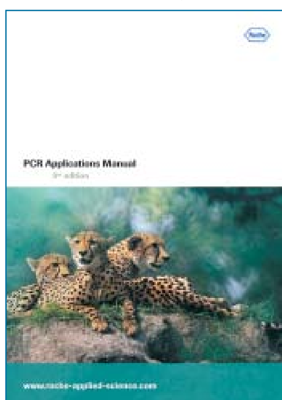


Figure 66: PCR Application Manual

Controls

Controls are essential for all important experiments. Depending on the experimental design, always select suitable controls and include them in all runs. The main types of controls that should be considered include:

Run Controls (various samples)

- Negative (NTC = *no template* control for each primer set)
- Positive (pre-tested samples; cloned plasmids; in relative quantification, a calibrator sample used for normalization of final results)
- Standards (for absolute quantification, defined genomic nucleic acid, plasmids, or PCR fragments with known target concentration; for mutation analysis, samples with known genotype)

Template/Sample Controls (various PCRs)

- RT (reverse transcriptase)-minus controls for RT-PCR, to detect DNA contamination in RNA sample preparations
- Internal control (usually a second artificial PCR with primers, probe(s) and template; co-amplified with the target to check for presence of reaction or detection inhibitors)
- Reference or housekeeping genes (detection of a second, stably expressed endogenous control gene, for determination of relative ratios and correction for sample-to-sample variations)

Replicates (identical set-up)

- PCR replicates (repeated, identical samples, to establish assay reproducibility or statistical data, especially for assay of samples which contain only low copy numbers of target)
- Biological replicates (duplicate samples, undergoing the entire nucleic acid purification process in an identical way, to cross-check for assay significance).

<table>
<tr><th rowspan="2">Run Controls</th><td>negative</td><td colspan="4">positive</td></tr>
<tr><td>no template control (NTC)</td><td>positive control</td><td colspan="2">calibrator</td><td>standard</td></tr>
<tr><th>Template/ Sample Controls</th><td>RT-minus</td><td colspan="2">internal</td><td colspan="2">reference</td></tr>
<tr><th>Precision Controls</th><td colspan="5">replicates</td></tr>
</table>

Master Mixes

Roche Applied Science offers a selection of dedicated, ready-to-use LightCycler® 480 master mixes, specially designed to support each of the main Real-Time PCR applications. These LightCycler® 480 reagents contain an improved hot-start PCR enzyme formulation, providing exceptional sensitivity and specificity in fast ramping PCR protocols. Furthermore, they offer extended room temperature stability for maximum robustness in automated workflows, and improved storage conditions for added convenience in daily use.

Key benefits of LightCycler® 480 System reagents:

- ▶ Enjoy exceptional detection sensitivity and specificity for standard and fast PCR protocols.
- ▶ Get maximum enzyme stability for automated high-throughput workflows at room temperature.
- ▶ Save time with ready-to-use, all-in-one master mixes.

Reagents	Formats	Applications	Hot-start PCR	PCR Speed	
				Standard	Fast
LightCycler® 480 SYBR Green I Master (2× concentrated)	SYBR Green I	Qualitative/ Quantitative	✔	✔	✔
LightCycler® 480 High Resolution Melting Master (2× concentrated)	ResoLight dye	Qualitative/ Quantitative	✔	✔	✔
LightCycler® 480 RNA Master Hydrolysis Probes	Hydrolysis probes, UPL probes	One-Step qRT-PCR	✔	✔	✔
LightCycler® 480 Probes Master (2× concentrated)	Hydrolysis probes, UPL probes, HybProbe probes, SimpleProbe probes	Qualitative/ Quantitative	✔	✔	✔
LightCycler® 480 Genotyping Master* (5× concentrated)	HybProbe probes SimpleProbe probes	Melting curve-based genotyping	✔	✔	✔

* Optimized for multiplex applications. Not suitable for end-point genotyping with hydrolysis probes because enzyme lacks 5'-exonuclease activity. ■ Sequence-independent DNA detection ■ Sequence-specific DNA detection

Figure 68: Application areas of the LightCycler® 480 reagents. All LightCycler® 480 reagents prevent carry-over contamination by employing dUTP for UNG (Uracil-DNA-Glycosylase)-mediated decontamination. For DNA masters, extended storage conditions enable storage at +4 to +8 °C for up to four weeks, in addition to the usual long-term storage conditions at -15 to -25 °C.

Reaction Conditions

A key variable is the $MgCl_2$ concentration, which can strongly affect the binding behavior of the PCR primers. Commercially available master mixes have already been optimized, and can often produce robust assays without requiring $MgCl_2$ titration.

To evaluate new applications, we recommend starting with a final primer concentration of 0.5 µM, a probe concentration of 0.2 µM, and a moderate amount of template NA (*e.g.*, 50 pg - 50 ng genomic DNA).

Variable salt concentrations in different samples, if carried into the amplification reaction, may influence the melting behavior of the PCR product. Therefore, try to make the salt level uniform in all samples, especially for HRM experiments.

Universal ProbeLibrary

Universal ProbeLibrary assays can be easily designed, yet have a high success rate. Optimization is usually not required. All amplification reactions follow a standardized PCR protocol. UPL probes can be combined with Universal ProbeLibrary Reference Gene Assays to quantify expression levels of a gene of interest relative to levels of an endogenous gene.

RealTime *ready* PCR Assays

RealTime *ready* Focus Panels are ready-to-use, pre-tested qPCR assays targeting a broad range of selected genes from specific pathways or functional groups. Conveniently supplied on LightCycler® 480 96- or 384-well plates, each assay contains target-specific primers and a Universal ProbeLibrary probe – all you need to add is master mix, water, and sample cDNA. Each panel is optimally designed for expression profiling of genes that play a major role in the respective pathways or functional groups (*e.g.*, cell cycle, GPCRs, nuclear receptors). The gene selections included on the panels have been carefully chosen in cooperation with experts in related fields of research.

In addition to the pathway- or functional-group-specific targets, each plate contains reverse transcription-specific controls and reference gene assays to facilitate data interpretation (see box below for an example). The convenient plate-based assays require only the addition of sample cDNA and master mix. Plate configuration details can be accessed online and downloaded to the LightCycler® 480 sample editor software.

As an example, the RealTime *ready* Human Apoptosis Panel can be used to quantify the expression levels of 372 human apoptosis-related genes directly on a LightCycler® 480 Instrument. The panel consists of 372 pre-tested, ready-to-use qPCR assays, supplied in a LightCycler® 480 384-well plate. Each assay includes the appropriate primers and a short hydrolysis probe that contains Locked Nucleic Acid (LNA). The only components that need to be added are sample cDNA and reaction mix (*e.g.*, LightCycler® 480 Probes Master).

In addition to the apoptosis-related assays, the plate contains seven human reference gene assays (ACTB, β2M, GAPD, HPRT1, RPL13A, 18s, and YWHAZ), which enable relative quantification of the apoptosis-related genes. The plate also contains three positive- and two negative-control assays for the reverse transcription (RT) reaction. The RT positive control is used to determine if degradation of your initial RNA sample has occurred and to assess the quality of your RT step. The RT negative control detects residual genomic DNA.

3

Standard Protocols

The LightCycler® System offers universal PCR run protocols for most assay formats (see Figure 67 below). These so-called template protocols are available in the LightCycler® 480 Software and can often be used "as is". However, they may need to be adapted for specific applications. Depending on primer design, optimal thermal cycling conditions (especially the annealing temperature) can vary from assay to assay. Refer to the technical data sheets provided by the oligo supplier to determine the optimal annealing temperatures. The optimal elongation time can be calculated using the formula:

elongation time = amplicon length (bp) ÷ 25.

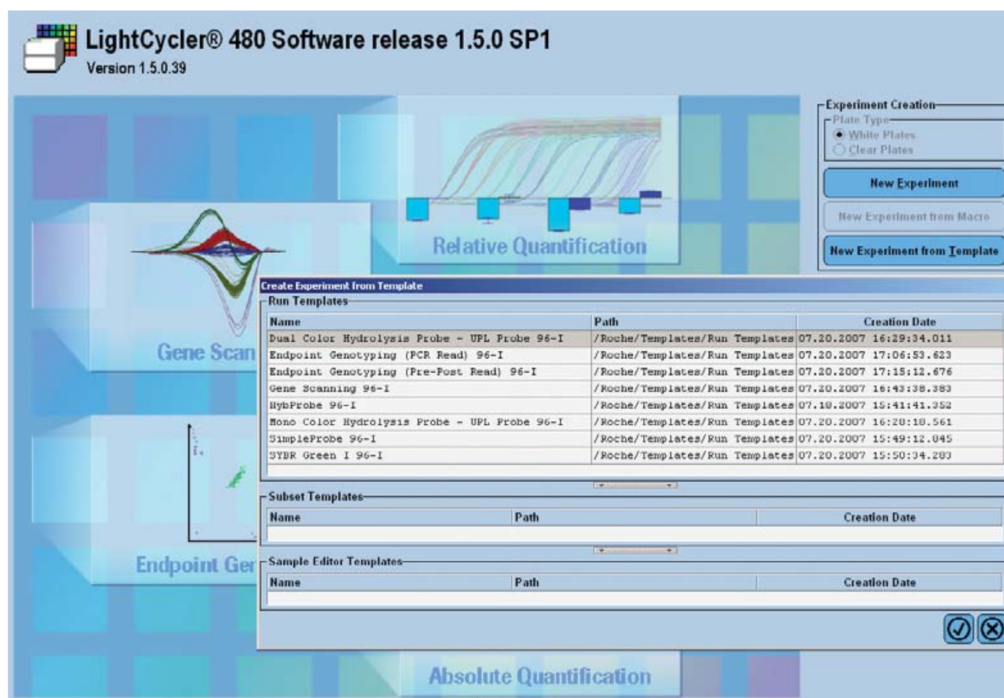


Figure 67

Special Considerations for Reverse Transcription PCR

Sample preparation is one of the most critical aspects of quantitative PCR. For best results, start with high quality RNA, *e.g.*, undegraded total RNA containing no inhibitors, no protein, and only a minimal amount of contaminating DNA. RNA isolation should always be performed carefully. Be aware that RNA is extremely susceptible to degradation by RNases present in all living cells, and that mRNA expression profiles might vary with the cell cycle and change after sample collection.

We recommend using commercially available kits or instruments to obtain highly pure and intact RNA, free of PCR inhibitors, *e.g.,* the Roche MagNA Pure LC or MagNA Pure Compact Systems for automated nucleic acid purification, or Roche High Pure Isolation kits for manual purification. Before isolating nucleic acids, you must completely homogenize the source tissue (*e.g.*, with the Roche MagNA Lyser).

One-Step or Two-Step RT-PCR

For PCR, RNA must be transcribed into cDNA. There are two strategies that combine reverse transcription and PCR. One-step RT-PCR reduces handling and analysis time, but requires separate reverse transcription reactions for each RNA studied. Two-step RT-PCR separates reverse transcription and PCR into two reactions. With either method, you can usually obtain enough cDNA from 1 µg total RNA.

Advantages of one-step RT-PCR:

- One tube assay (easy handling, low risk of contamination)
- Fast
- Requires target-specific priming

Product Recommendation: LightCycler® 480 RNA Master Hydrolysis Probes

This master is an easy-to-use hot start reaction mix, specifically adapted for one-step RT-PCR in the plate-based LightCycler® 480 Instruments, using the hydrolysis probe (*e.g.*, UPL) detection format.

Advantages of two-step RT-PCR:

- Multiple PCRs from one RT reaction, derived from a common cDNA pool
- Different options for choice of RT primers (oligo dT, random hexamers, target-specific)
- cDNA can be stored for long periods of time

Product Recommendation: Transcriptor High Fidelity cDNA Synthesis Kit

- Achieve high sensitivity – reverse transcribe from as little as 10 pg template RNA; simultaneously reverse transcribe both rare and abundant RNAs without altering gene expression levels.
- Increase accuracy – achieve 7-fold higher fidelity compared to normal reverse transcriptases.
- Obtain full-length cDNA with high yields – transcribe up to 14 kb with the anchored-oligo(dT)18 primer.
- Get results faster – obtain full-length cDNA synthesis in only 10 min.
- Power through all kinds of RNA templates – effectively reverse transcribe normal and GC-rich RNAs at incubation temperatures up to 55°C.

For optimal RT-PCR sensitivity, design PCR primers and/or probes (*e.g.*, UPL) that span exon-intron boundaries and limit amplicon length to < 200 bp.

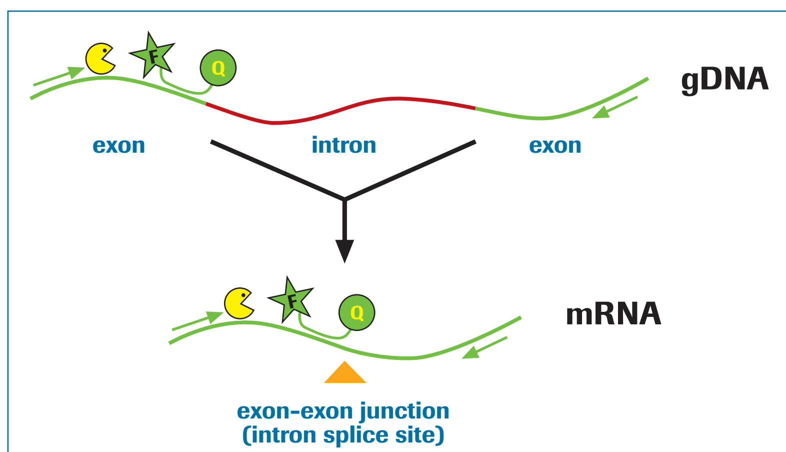


Figure 68: Intron-spanning assay design prevents problems with background from genomic DNA contamination.

RNA Pre-Amplification

For some gene expression studies, sample material is often difficult to obtain and very limited in quantity. In this case, to achieve a good understanding of gene regulation, a single sample must be used to screen for many transcripts, requiring more sample than is available. To overcome this bottleneck when using very low amounts of sample, it is possible to carry out pre-amplification of RNA. One solution is pre-amplification of RNA.

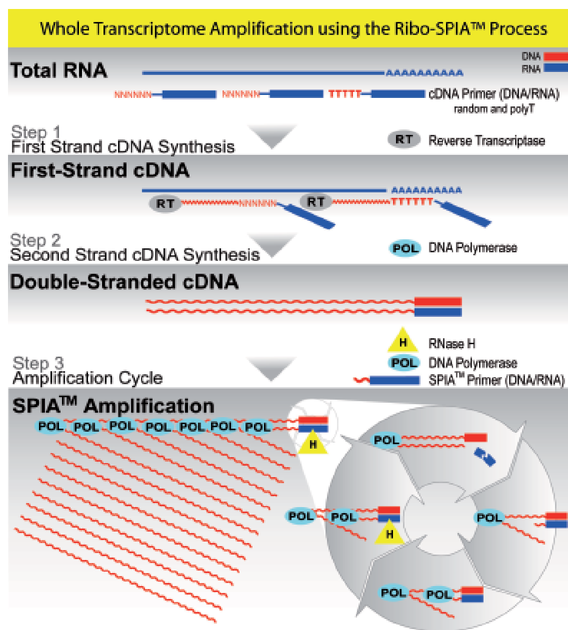


Figure 69: Principle of RNA pre-amplification using the LightCycler® RNA Pre-Amplification Kit (Ribo-SPIA® technology).

The **LightCycler® RNA Pre-Amplification Kit** uses a rapid, simple and sensitive amplification process (Ribo-SPIA® technology):

First Strand Synthesis

First strand cDNA is prepared from total RNA using a First Strand Primer Mix (DNA/RNA chimeric primer mix) and Transcriptor Reverse Transcriptase (RT). The primers have a DNA portion that hybridizes either to the 5´- portion of the poly (A) sequence or randomly across the transcript avoiding to create a bias in first strand synthesis. The RNA portion of the primer will help create a priming site for the following amplification step.

Second Strand Synthesis

After fragmentation of mRNA in the cDNA/mRNA complex, DNA polymerase generates a second strand, using the first strand cDNA as template. This new cDNA strand has the same sense orientation as the original RNA. The result is a double-stranded cDNA with a DNA/RNA heteroduplex at one end. So it contains a short, single-stranded RNA sequence (from the original chimeric primer) on one end.

Isothermal Linear Amplification

The Ribo-SPIA® Amplification is a linear isothermal DNA amplification process that uses a SPIA® DNA/RNA chimeric primer, DNA polymerase and RNase H in a homogeneous isothermal assay that provides highly efficient amplification of DNA sequences. RNase H is used to degrade RNA in the DNA/RNA heteroduplex at the 5´- end of the first cDNA strand. This results in the exposure of a DNA sequence that is available for binding a second SPIA® DNA/RNA chimeric primer. DNA polymerase then initiates replication at the 3´- end of the primer, displacing (but not destroying) the original, first-strand cDNA. RNase H then cleaves the RNA at the 5´ end of the new strand, partially exposing the priming site for the next round of cDNA synthesis. The entire process (chimeric priming, then cDNA synthesis, then RNase H cleavage) repeats continuously and always copies the same cDNA molecule. The result is the rapid accumulation of amplified single-stranded antisense cDNA products, which are complementary to the original RNA. An average amplification of 1,500-fold is observed with 5 ng starting total RNA. Amplified cDNA product is ready for qPCR and does not require purification.

Evaluation of PCR Assays

Remember that the reliability of all Real-Time PCR applications depends on the quality of the PCR. If you follow the previously discussed recommendations for assay design and PCR set-up, you can rapidly and efficiently optimize a Real-Time PCR assay. A perfectly optimized assay will display little or no test-to-test variation in crossing point and fluorescence signal intensity. An optimized assay will have steep amplification curves, high signal intensities and low crossing points (C_p). Such assays will also show the expected results for both negative and positive controls.

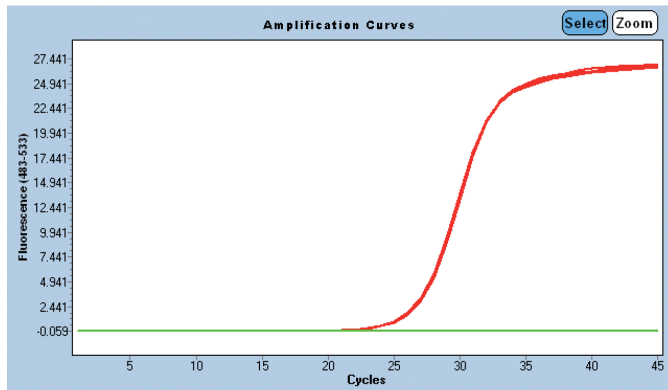


Figure 70: An ideally optimized PCR amplification curve.

3

Important criteria for an optimized PCR assay are:

- Specificity
- Efficiency
- Sensitivity
- Reproducibility

Specificity

The specificity of PCR depends mainly on accurate primer design. Be aware that SYBR Green I assays detect all double-stranded DNAs, including primer dimers. Avoid nonspecific products by carefully selecting primer and reaction conditions. Suitable test systems for verifying specificity of amplification are melting curve analysis and agarose gel electrophoresis.

Probe-based assays are sequence-specific; nevertheless, both primer and probe must be designed properly if the assay is to be successful.

Negative and positive controls are essential for verifying the specificity of a PCR assay.

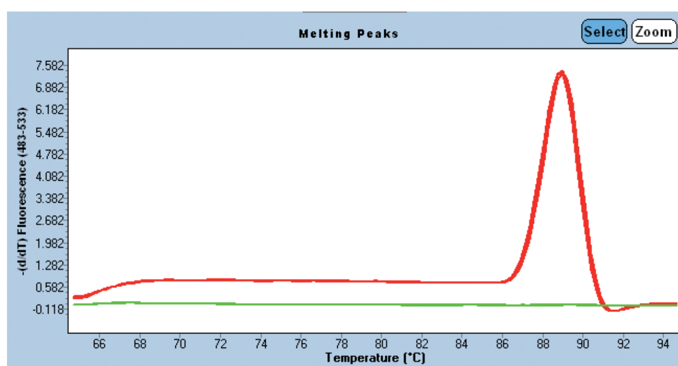


Figure 71

3

Efficiency

How well a Real-Time PCR assay works for a specific parameter and sample type is usually determined by the efficiency of the PCR. Efficiency is a quantitative expression of the quality, activity, or effectiveness of the PCR process. In other words, PCR efficiency provides a good estimate of how completely the amplicon is amplified during each cycle. The highest quality PCRs run with an efficiency of 2, meaning that the number of target molecules doubles with every PCR cycle. Unfortunately, PCR efficiency is highly sensitive to many aspects of the reaction that can vary from run to run (*e.g.*, sample preparation, PCR primers). Consequently, two different PCRs (*e.g.*, for a target gene and a housekeeping gene) may not have identical efficiencies.

To double or not to double, that´s the question!

Suboptimal efficiency of PCR is a likely explanation for low detection rates, low assay sensitivity or insufficient assay reliability and accuracy. Not all amplification reactions can be optimized to achieve perfect doubling. For example, templates with high GC content or other so-called difficult templates may reduce PCR efficiency. Nevertheless the PCR should be optimized to the highest possible efficiency, to ensure a stable reaction with high reproducibility.

A "reasonable" PCR should be >85% efficient. There is some ambiguity in how people define efficiency. Some argue that if you copy 90% of your DNA in a cycle, so that you end up with 1.8 times as much, the efficiency is 1.8. Others say that the efficiency is actually 0.8 since one makes 0.8 times as much. If you use this definition the fold increase will be $[1 + \text{efficiency}]^n$. We have adopted the first version, so we do not have to keep adding the '1'. An efficiency of 2.0 therefore represents a 100% doubling, 1.9 represents a 95% efficiency, and so on.

Factors Influencing PCR Efficiency

There are several factors that influence PCR efficiency; most of them discussed in previous chapters.

Efficiency is highly dependent on basic PCR conditions such as the length, the G/C content of the amplicon and secondary structure.

Even the dynamics of the reaction itself can interfere with efficiency. Variations in the dynamics can result from the enzymes used in the reaction and/or non-optimal reagent concentrations.

Very often, poor primer quality is the main cause for poor PCR efficiency. In this case, the PCR amplification curve usually reaches plateau early and the final fluorescence intensity is significantly lower than that of most PCRs. To solve this problem, re-synthesize the primers.

Determination of PCR Efficiency

PCR efficiency can be easily determined in any Real-Time PCR instrument. There are several methods available and these have been discussed frequently in the scientific community.

For highly standardized and reproducible calculation of PCR efficiency that is completely independent of the detection sensitivity, we recommend the well-established and well-accepted standard curve method, which in some publications is also called the "Calibration Curve Method".

The standard curve method is an excellent tool for examining the overall quality of a PCR assay. A standard curve is a plot of the cycle number at the crossing point (Y-axis) versus the log of initial template amount (X-axis), derived from an assay based on serial dilutions. The standard curve is a least square fit line drawn through all dilutions.

Serial dilutions should be based on a standard sample with a well-characterized absolute concentration of the target, or on a positive sample diluted in defined steps (*e.g.*, 1:10, 1:100, etc.) that represent relative concentrations (expressed in arbitrary units) of the target.

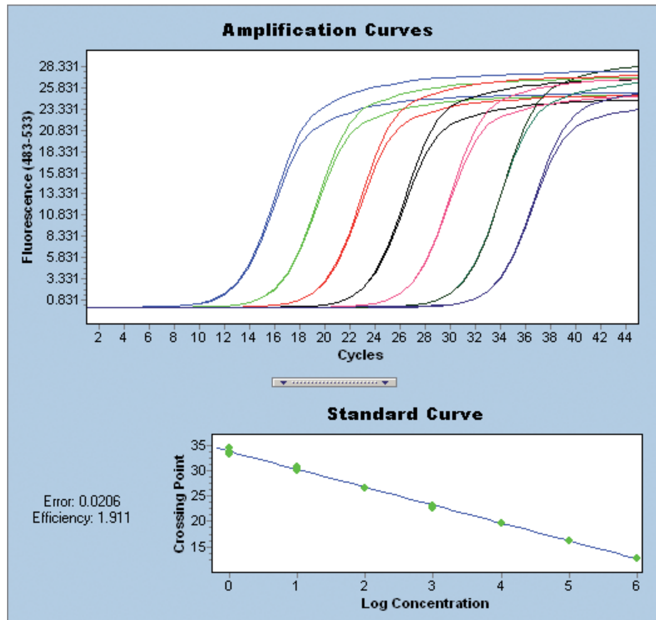
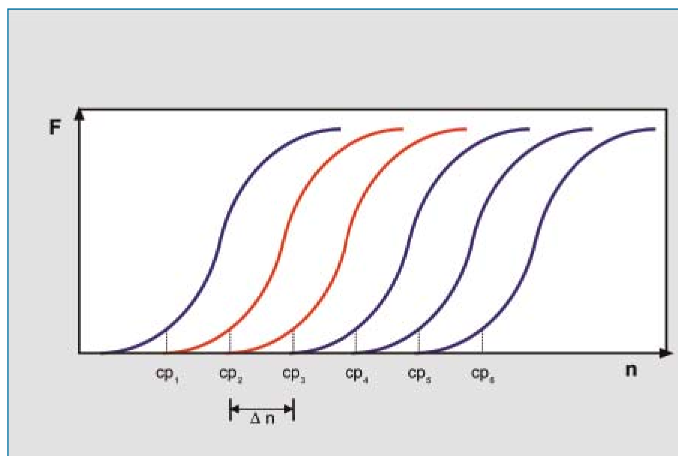


Figure 72: Quantification standard curve obtained by serial 1:10 dilutions.

The PCR efficiency can be directly calculated from the slope of the standard curve (= distance from the C_p of one dilution to the next) by using the formula:

$$E = 10^{-1/slope}.$$

Samples that differ by a factor of 2 in their original DNA concentration are expected to have C_p values 1 cycle apart; thus the C_p of samples that differ by a factor of 10 are ~3.3 cycles apart (if PCR efficiency is 2).



for 10-fold dilutions:

$\Delta n = 1 / \log E$

e.g. $E = 2.0$ $\Delta n = 3.32$

Figure 73

Efficiency testing should be done across a broad template dilution range to determine the sensitivity limits of an assay. The regression line of the standard curve will serve as an indicator of the consistency of the reaction over the whole range of template concentrations. The error value (displayed next to the plot in the results) is a parameter that reflects how well all data points fit the regression line.

If replicates of each serial dilution are assayed, the precision of the standard curve can be determined; this parameter reflects assay reproducibility and accuracy. Repeating the standard curve run will help you gain further confidence in the stability of the assay.

3

Sensitivity

A positive amplification curve indicates the presence of a specific target DNA. The higher the concentration of target in the starting material, the sooner a significant increase in fluorescent signal will appear, yielding a lower C_p.

Well-optimized PCR assays allow detection of a target in low amounts of template nucleid acid. The ultimate level of sensitivity will allow amplification and detection of one copy of target; this level can easily be achieved by highly efficient PCRs. Be aware that results with values near the detection limit of an assay (<100 copies) should be based on statistical evaluation of replicate samples, since at low template concentrations, particle distribution can affect results.

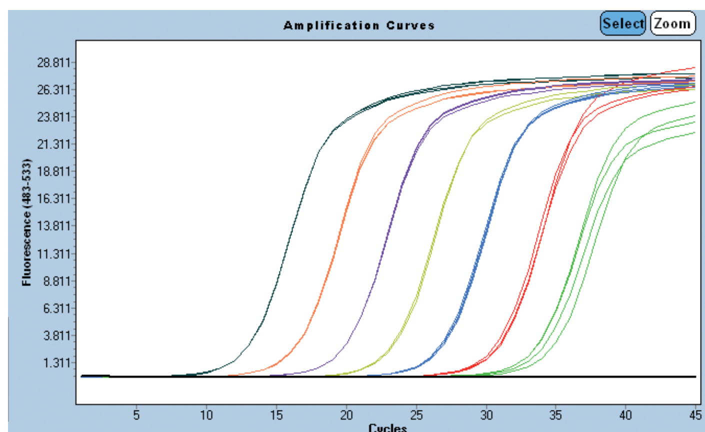


Figure 74

Dynamic Range

Here again, a standard curve is an excellent tool for demonstrating the dynamic range and the detection limits of an assay. To determine range, start with the highest possible concentration of one typical positive sample, then dilute in *e.g.*, 1:10 steps, down to the detection limit of the assay. Good assays can easily cover a dynamic concentration range of 4 - 6 orders of magnitude. The LightCycler® System can accurately monitor template concentrations over 10 orders of magnitude, which is important for detecting viral loads and plasmid copy numbers.

Determination of Crossing Points (C_p)

A standard curve plots crossing point versus the log of template concentration of a diluted standard/ sample. To guarantee consistent performance and reliability especially for determination of C_p values, an automatic, user-independent software algorithm is important. This algorithm needs to be robust, yet at the same time sensitive, to be able to handle data at the detection limit or data derived from non-linear assays (since not all amplification reactions follow a linear regression throughout the entire assay).

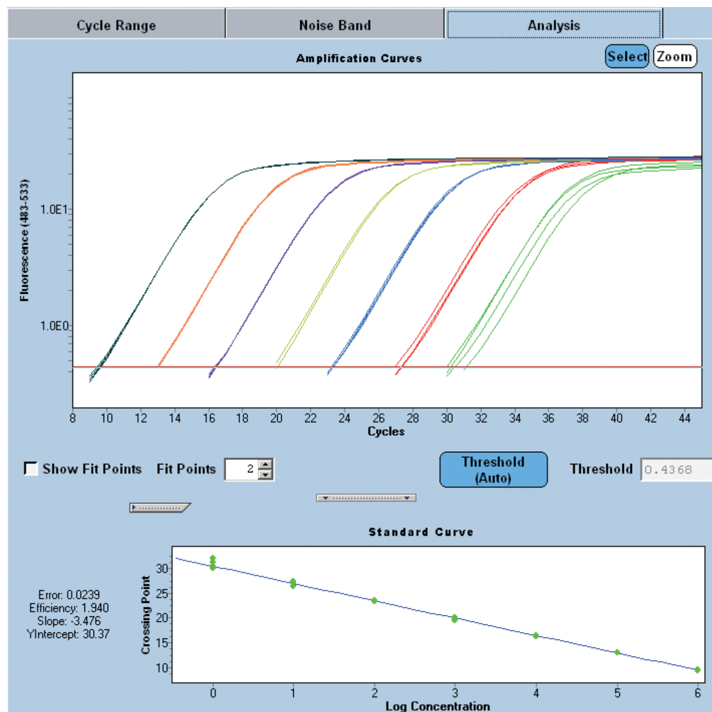


Figure 75

3

The LightCycler® Data Analysis Software provides two algorithms for the determination of crossing points: the Fit Points Method and the Second Derivative Maximum Method. The two methods differ in the way the crossing point (where the fluorescence signal first exceeds the level of background noise) is calculated. However, regardless of the absolute value of the resulting C_ps, these methods are equally suitable for accurate evaluation; within a single run, results for unknown samples as well as standards are calculated by the same algorithm. Briefly, here is how these two methods work:

- The **Fit Points Method** requires adjusting baseline, setting the noise band, and entering the number of data points to be considered in the log-linear phase. In LightCycler® 480 Software, Version 1.5, analysis is fully automated. However, fine-tuning adjustments can be made manually by the user.
- The **Second Derivative Maximum Method** offers fully automated data calculation, with no user input, based solely on the shape of the curve. The C_p value is determined by identifying the first maximum of the second derivative of the amplification curve. This method offers the advantages of speed and simplicity, especially for highly reproducible analysis of data coming from different runs or different users.

  Even if weak, non-linear signals occur at low concentrations, the software will automatically plot curves that fit all data. The standard curve plot "C_p vs. log concentration" is then displayed as a non-linear curve instead of a straight regression line, thus allowing accurate analysis, even for results close to the detection limit.

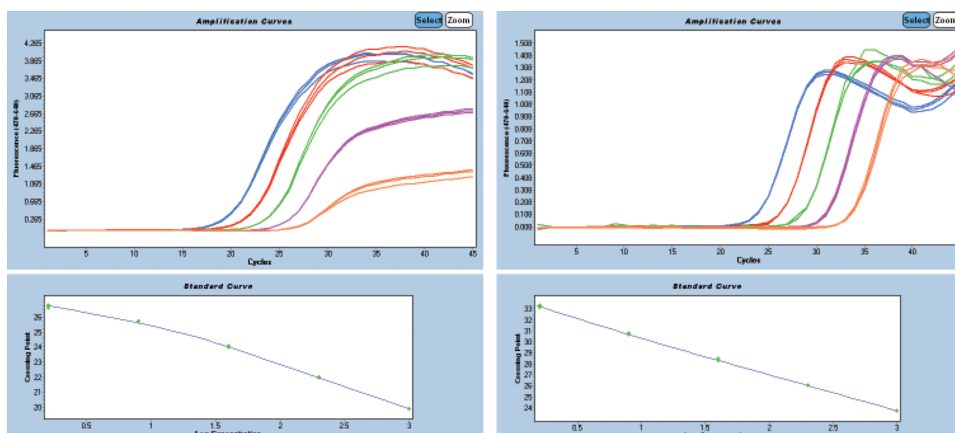


Figure 76

3

Reproducibility

Generating Precise Standard Curves

To correctly interpret results of Real-Time PCR measurements, it is necessary to determine the PCR efficiency precisely.

The figure below shows standard curves derived from two amplification systems with different efficiencies. Since the two standard curves are neither identical nor parallel, the values assigned to two unknown samples changes depending on which curve is used.

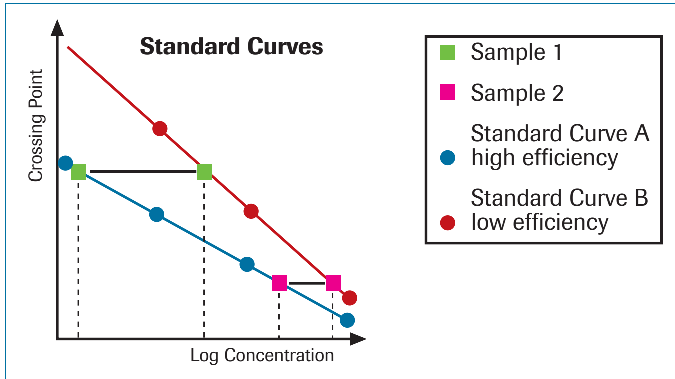


Figure 77: Precise Standard Curves

A prerequisite for accurate efficiency determination is a stable, reproducible PCR run, independent of sample material and background matrix. In general, carefully optimized PCRs (with respect to PCR program, reaction components, primer design, etc.) lead to highly reproducible runs.

- **Variation is low down to 100 copies.**
- **Below 100 copies the CV increases due to particle distributuion**

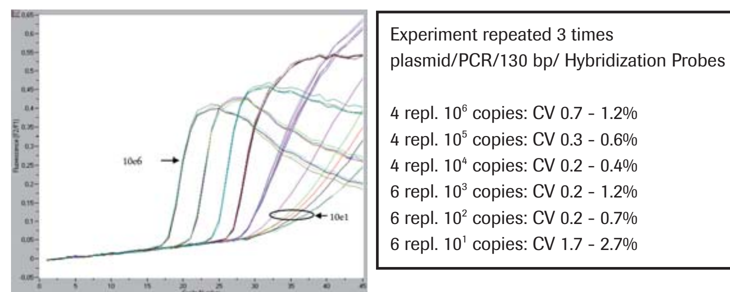


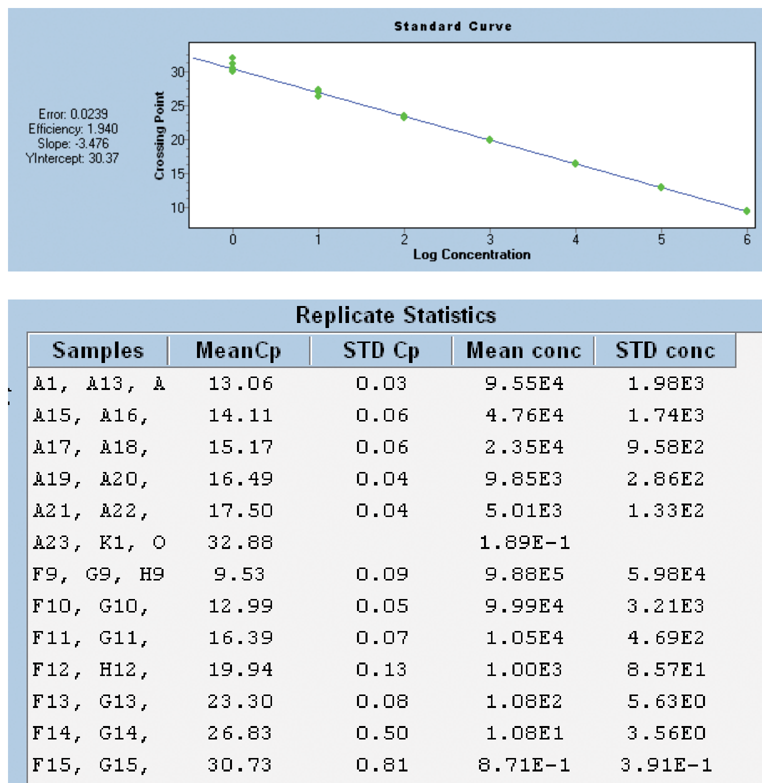
Figure 78

Statistical Considerations

We recommend establishing an appropriate statistical basis for a standard curve. In other words, do not use a so-called “three-point standard curve.” Instead, run enough data points to create a statistically valid standard curve.

To meet statistical requirements, the standard dilutions should span at least 3–5 orders of magnitude, and all dilutions should fall within the expected concentration range of the unknowns to be analyzed. In addition, use a minimum of 4–5 serial dilutions (*e.g.*, 1:10 dilutions) for each curve and prepare 3–6 replicates of each dilution. A measure of precision for a standard curve is given by the error value (mean squared error), which indicates how well the individual data points fit the regression line. Replicates are especially important for lower concentrations, since the C_v (coefficient of variation) at low concentrations increases due to particle distribution.

The figure below shows an example of a standard curve that is highly reproducible, and has a standard deviation (STD of C_p) < 0.1 down to values of 10-100 copies.



Replicate Statistics

Samples	MeanCp	STD Cp	Mean conc	STD conc
A1, A13, A	13.06	0.03	9.55E4	1.98E3
A15, A16,	14.11	0.06	4.76E4	1.74E3
A17, A18,	15.17	0.06	2.35E4	9.58E2
A19, A20,	16.49	0.04	9.85E3	2.86E2
A21, A22,	17.50	0.04	5.01E3	1.33E2
A23, K1, O	32.88		1.89E-1	
F9, G9, H9	9.53	0.09	9.88E5	5.98E4
F10, G10,	12.99	0.05	9.99E4	3.21E3
F11, G11,	16.39	0.07	1.05E4	4.69E2
F12, H12,	19.94	0.13	1.00E3	8.57E1
F13, G13,	23.30	0.08	1.08E2	5.63E0
F14, G14,	26.83	0.50	1.08E1	3.56E0
F15, G15,	30.73	0.81	8.71E-1	3.91E-1

Figure 79

Multiple Plate Analysis Software

Managing an increasing amount of information requires additional tools that can compile, visualize and subject data to statistical calculations.

A tool that can help with such analyses is the LightCycler® 480 Multiple Plate Analysis Software, which is offered as an accessory module to the LightCycler® 480 Software, Version 1.5. This module can analyze data from multiple experiments, *e.g.*, for statistical comparison of data over an extended period of time. It can offer large-scale comparisons for all types of analysis available on the instrument (*i.e.*, absolute and relative gene quantification, melting curve analysis, genotyping, endpoint genotyping and gene scanning).

LightCycler® 480 Multiple Plate Analysis Workflow Overview

In LightCycler® 480 Software, Version 1.5:

- Analyze data
- Export data as *.ixo files

In the Multiple Plate Analysis Software:

- Import *.ixo files into SQL database
- Create study
- Assign experiments to the study
- Define grouping criteria
- Assign samples to groups for comparison
- Select analysis mode
- Create analysis

Results are displayed on automatically generated spreadsheets.

It is easier to evaluate experimental results, when these data show a certain trend, indicate a certain stringency or have properties based on certain values. The Multiple Plate Analysis Software allows optional grouping of data and therefore can help with such evaluations:

- It allows you to create groups by assigning properties to the samples (*e.g.*, gender, behavior, …).
- It can then compare results from the different groups (*e.g.*, treatment 1 vs. treatment 2).

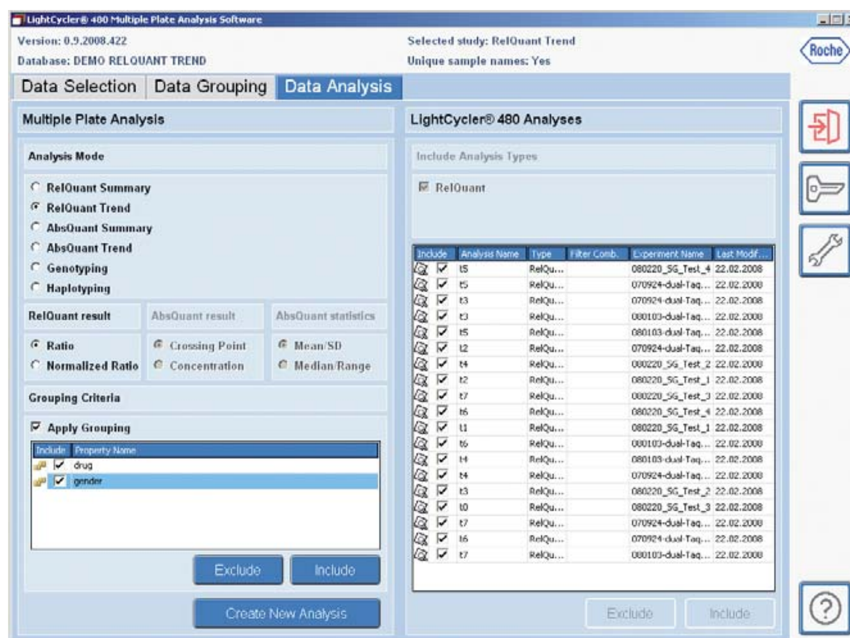


Figure 80: Setting up a multiple plate analysis.

For example, the *Statistics* sheet can compare different samples according to their classification. It contains the *Statistics* graph (Box Plot View) and the *Mean Value and Standard Deviation* table for all samples assigned to a study. The information shown in the graph and table depends on the analysis mode and the options selected on the *Data Analysis* tab. By default, the values for all samples are displayed. To limit the samples to be taken into account, simply click *Filter* and assigning filtering criteria.

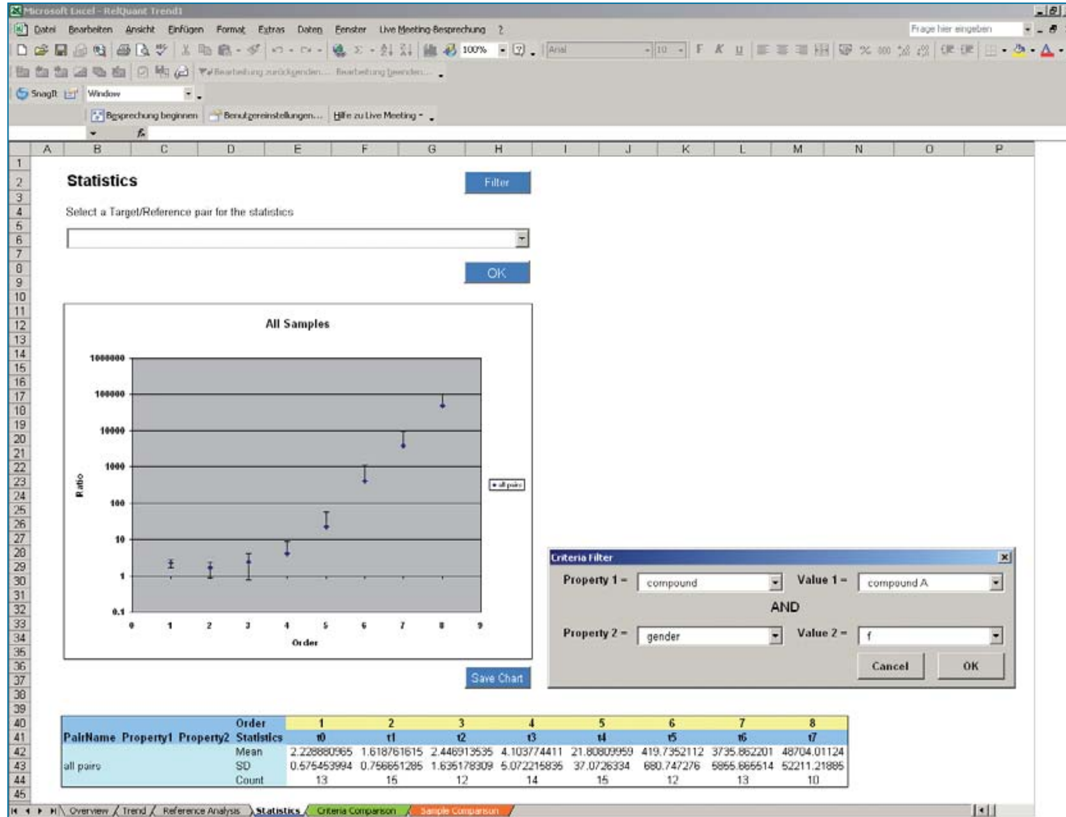


Figure 81: Applying filters to a multiple plate analysis result.

Four major types of analysis are available:

- *Summary* – available for both Absolute and Relative Quantification.
- *Trend* – displays data trends for different research samples and/or targets, *e.g.*, changes in RNA expression that depend on incubation with an active compound.
- *Genotyping* – used to display the genotypes of research samples in different analyses and to calculate the allele frequency of variants.
- *Haplotyping* – used to display and count the distribution patterns for variations on the same chromosome.

The results of the data analyses are displayed in spreadsheets and/or charts, which are similar to those commonly found in Microsoft Excel. Therefore, these data can easily be exported to Microsoft Excel for further analysis.

For a practical example of how the LightCycler® 480 Multiple Plate Analysis Software was used in a gene expression research project, please see the article on page 96.

Analysis Mode	Input	Derived from LC480 SW 1.5 Analysis	Output into Excel	
AbsQuant Summary	C_p values or concentrations	AbsQuant/2nd Derivative or AbsQuant/Fit Points	overview summary analysis box plot analysis t-Test	analysis comparison criteria comparison criteria t-Test sample comparison
AbsQuant Trend	C_p values or concentrations	AbsQuant/2nd Derivative or AbsQuant/Fit Points	overview trend statistics	criteria comparison sample comparison
RelQuant Summary	ratio or normalized ratio	RelQuant Basic or Advanced	overview summary analysis box plot analysis t-Test	analysis comparison criteria comparison criteria t-Test sample comparison
RelQuant Trend	ratio or normalized ratio	RelQuant Basic or Advanced	overview trend reference analysis	statistics criteria comparison sample comparison
Genotyping	genotype	Endpoint Genotyping Melt Curve Genotyping Gene Scanning	overview results group names	genotyping Hardy-Weinberg criteria comparison
Haplotyping	genotype	Endpoint Genotyping Melt Curve Genotyping Gene Scanning	overview results group names	haplotyping criteria comparison

Practical Examples and Experimental Studies

4

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Quantitative Detection of *Legionella pneumophila* in Water Samples: Assay Transfer to the LightCycler® 480 Real-Time PCR System

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An assay to detect and quantify *Legionella spp.* in water samples was successfully transferred from a capillary-based LightCycler® Instrument to the novel multiwell plate-based LightCycler® 480 System. A previously described protocol was easily adapted by modifying the fluorescent label of the detection probe to match the filter set of the LightCycler® 480 Instrument. While reproducibility and analytical sensitivity of the assay were found to be comparable on both systems, the novel plate-based instrument allowed for higher sample throughput and reduced assay time per sample.

4

1 Introduction

Legionellae are gram-negative bacteria that are ubiquitous in environmental water sources and may cause sporadic as well as epidemic cases of atypical pneumonia. *Legionella pneumophila* is the most common pathogenic species, accounting for up to 90% of legionellosis cases. Diagnostic culture is considered the gold standard for the laboratory detection of *Legionella* infections, but successful culture requires selective media and prolonged incubation periods. As a consequence, nucleic acid amplification techniques can be evolved as a helpful complement to traditional diagnostic methods for detection and quantitation of *Legionella* DNA in clinical as well as in environmental samples [1].

We have previously described a sensitive and specific hybridization probe-based Real-Time PCR assay, allowing the simultaneous detection and quantification of *Legionella spp.* and *L. pneumophilia* with capillary-based LightCycler® Instruments [2]. The protocol includes amplification based on *Legionella* genus-specific primers flanking a species-specific segment within the 16S rRNA gene, followed by detection with a pair of LightCycler® Red 705-labeled hybridization probes complementary to a *L. pneumophila*-specific region within the amplicon.

The novel plate-based LightCycler® 480 System is an attractive platform for gene detection and quantification applications, because it maintains key characteristics of the capillary-based LightCycler® Systems (*e.g.*, speed, flexibility, sensitivity, and melting curve analysis) but allows for scale-up to higher reaction throughputs. The purpose of the presented study was to investigate how easily the existing *Legionella* DNA quantification assay could be transferred from the capillary to the plate-based system and which points in the procedure require special consideration.

2 Material and Methods

DNA preparation of environmental water samples

Legionella quantification standards were established, consisting of sterile water spiked with known amounts of *L. pneumophila* serogroup 1. One ml of each environmental water sample as well as the standards were filtered in a filtration device and the filter was placed in 2-ml caps. Following the manufacturer's instructions for the MagNA Pure LightCycler® DNA Isolation Kit III (Bacteria, Fungi), 460 µl of bacteria lysis buffer and 40 µl proteinase K were added to the filter and incubated on a shaking thermomixer for 20 minutes at 65°C, followed by an incubation step at 95°C for 10 minutes. Then the sample was allowed to cool to room temperature and transferred into the sample cartridge of the MagNA Pure LC Instrument. Five-microliter aliquots of the extracted DNA were directly transferred to the PCR mixture. The remainder of the DNA preparation was stored at -20°C for further experiments.

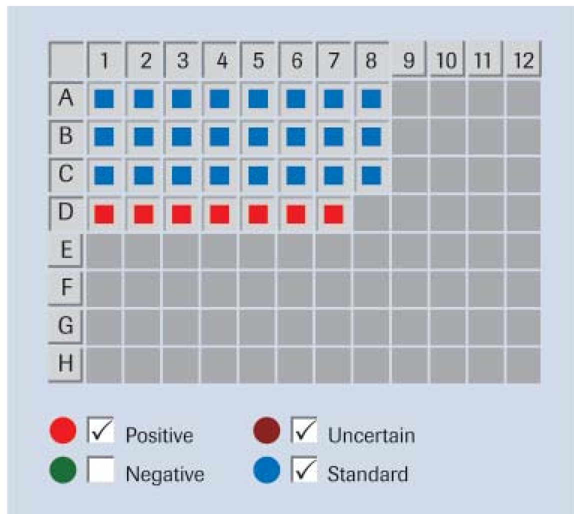

Figure 1: Plate set-up for quantification of *Legionella pneumophila* using the LightCycler® 480 System. Plate rows A, B, C (triplicates, shown in blue) contained standard samples in increasing concentrations (from 10^0 per reaction in column 1 to 10^7 per reaction in column 8). Wells in row D (red) contained unknown samples.

PCR primers and probes

The used oligonucleotide primers amplify a region of 386 base pairs within the 16S rRNA gene of *Legionella spp.*. The sequence of the forward primer was 5´-AGGGTTGATAGGTTAAGAGC-3´, that of the reverse primer was 5´-CCAACAGCTAGTTGACATCG-3´. The hybridization probes were located at a species-specific region for *L. pneumophila*. The sequences of the 3´ fluorescein-labeled and the 5´ LC Red 670, 3´phosphorylated hybridization probes were 5´-CCAGTATTATCTGACCGTCCCA-3´ and 5´-TAAGCCCAGGAATTTCACAGATAACTT-3´, respectively. The probes are positioned in such a way that a gap of three bases is left between them in order to allow efficient energy transfer between the two fluorophores. Primers and the hybridization probe oligonucleotides were synthesized by TIB MOLBIOL, Berlin.

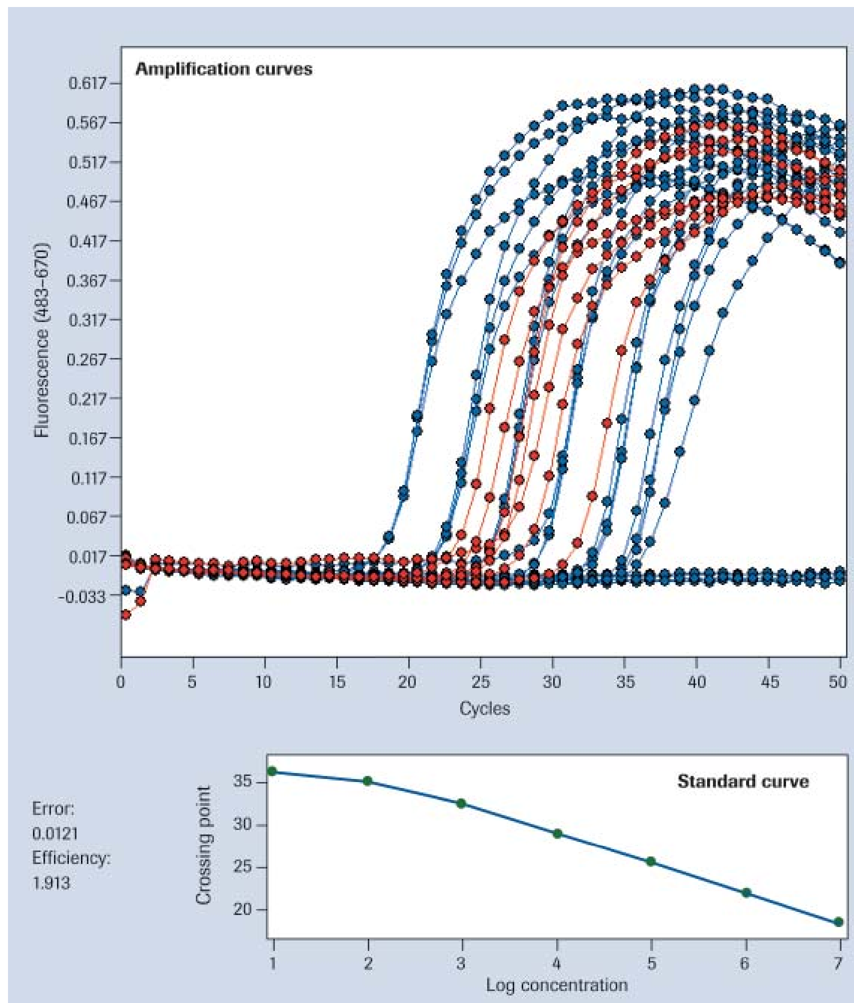

Figure 2: HybProbe probe-based real-time assay for *Legionella* quantification.
(a) Amplification curves obtained by measuring fluorescence of HybProbe probes in the 670 nm channel. Standards are shown in blue, unknown samples in red.
(b) Standard curve generated from known concentrations and Cp (crossing point) values of samples with known concentration.

PCR amplification

Rapid-cycling Real-Time PCR and melting curve analysis were performed with a LightCycler® 480 Instrument, Software v 1.1. The PCR mixture for the 16S rRNA gene hybridization probe assay contained 5 µl of sample DNA, 0.5 µM of each primer, 0.2 µM of each probe, 10 µl of LightCycler® 480 Probes Master, and PCR-grade sterile water to a final volume of 20 µl. Thermocycling conditions were as follows: 95°C for 10 minutes for initial denaturation and activation of Taq polymerase, followed by 50 thermal cycles of 95°C for 10 seconds, 50°C for 20 seconds, and 72°C for 30 seconds, with a ramping rate of 4.4°C/second, 2.2°C/second, and 4.4°C/second, respectively. Fluorescence was measured once during each 50°C step. Following the amplification process, a melting curve analysis was performed by heating the plates at 95°C for 20 seconds, incubating at 62°C for 20 seconds, followed by cooling to 40°C for 1 second and then slowly (with 5 acquisitions per °C) heating to 95°C. Fluorescence was monitored continuously during the melting experiment.

3 Results and Discussion

Detection and quantification of bacteria in aqueous samples can be performed based on amplification and melting curve analysis using HybProbe probes that bind to sequence regions unique for bacterial genera or species. To test if an existing *Legionella* quantification assay can be easily transferred from the LightCycler® 2.0 System to the LightCycler® 480 System, we used *L. pneumophila* as an example. Due to differences in detection filters available on both instruments (the LightCycler® 480 System includes a filter for detection at 500 nm while a 705 nm filter is not availble), the HybProbe sensor probe was used with the same sequence as before but relabeled with the LightCycler® Red 670 dye.

Analysis of amplification curves showed that reactions with standard samples had very high inter-well reproducibility (Figure 1 and Figure 2). When quantification results (Figure 3) were compared with results previously obtained using a capillary-based system, the sensitivity was found to be almost identical. We also included a post-PCR melting curve analysis to confirm PCR specificity and found that probe relabeling had only a minor effect on melting curve shape and Tm (data not shown). Due to the fact that the time required for melting curve analysis on a plate is independent of total sample number, the total running time (1 hour 55 minutes for multiwell plates, 1 hour 42 minutes for capillaries) was also nearly the same. Without the melting curve step, we would estimate the capillary-based system to be approximately 40 minutes faster. Nevertheless, with a plate-based system, at least three times more samples (96 or 384, compared with 32 in a capillary carousel) can be analyzed in parallel.

Both the LightCycler® 2.0 Instrument and the LightCycler® 480 Instrument are equipped with filter sets allowing for advanced assay set-ups (*e.g.*, dual color applications with one probe set detecting *Legionella* on genus level plus another probe set for specific detection of pathogenic species like *L. pneumophila*) [1]. When transferring this type of assay between systems, one must consider that the sets of available detection wavelengths are similar but not identical on different instruments. If probe relabeling is required, we recommend shifting both detection wavelengths in the same direction to reduce channel crosstalk, by moving for example from a 640 nm/705 nm channel combination with the LightCycler® 2.0 Instrument to 610 nm/670 nm with the LightCycler® 480 Instrument. Based on our experience, we expect the efforts associated with revalidating the assays after shifting wavelengths to be manageable.

In the present study, we found intra-assay variations (*e.g.*, standard deviations of Cp values) with the LightCycler® 480 plates to be at least as low as those generally observed between different LightCycler® capillaries. This makes it possible to achieve highly accurate results with fewer sample replicates (*e.g.*, duplicates instead of triplicates), for example when establishing standard curves for absolute quantification. Together with the multiwell, high-throughput characteristics of the LightCycler® 480 Instrument, this contributes to a reduced assay cost per data point.

Samples				Results		
Include	Color	Pos	Name	Cp	Concentration	Stand...
✓	■	D1	L06-03.15.0156	26.82	4.08E4	
✓	■	D2	L06-03.15.0157	27.78	2.19E4	
✓	■	D3	L06-02.10.0686	24.8	2.42E5	
✓	■	D4	L06-02.05.0226	31.07	2.59E3	
✓	■	D5	L06-02.05.0227	25.59	9.05E4	
✓	■	D6	L06-01.27.0526	25.99	6.99E4	
✓	■	D7	L06-01.12.0259	23.04	4.72E5	
✓	■	D8	Negative control			

Statistics				
Samples	Mean Cp	STD Cp	Mean conc	STD conc
A1, B1, C1				
A2, B2, C2	36.14		1.06E1	
A3, B3, C3	34.83	0.34	1.01E2	4.98E1
A4, B4, C4	32.58	0.20	9.78E2	1.31E2
A5, B5, C5	28.82	0.02	1.11E4	1.70E2
A6, B6, C6	25.47	0.03	9.79E4	1.70E3
A7, B7, C7	21.96	0.02	9.56E5	9.41E3
A8, B8, C8	18.34	0.07	9.98E6	4.68E5

Figure 3: Results of concentration determination for samples D1 to D8 (above) and standard curve triplicates (below). STD: standard deviation.

4 Conclusions

The novel LightCycler® 480 Real-Time PCR System was found to be a highly suitable multiwell plate-based system for quantification of *L. pneumophila* DNA in environmental water samples. An assay established previously with a capillary-based LightCycler® System was seamlessly transferred and scaled up without significant losses in sensitivity or speed. Depending on their workflows (*e.g.*, sample throughput, automation requirements), laboratories now have the choice between two systems that can simultaneously detect and differentiate bacterial species based on mono- or dual-color protocols.

5 References

1. Wellinghausen N *et al.* In: Reischl U, Wittwer C, Cockerill F (eds) (2001) Rapid Cycle Real Time PCR - Methods and Applications: Microbiology and Food Analysis. Springer Verlag, Heidelberg
2. Reischl U *et al.* (2002) J Clin *Microbiol* **40**: 3814–3817

Ordering Information

Product	Cat. No.	Pack Size
LightCycler® 480 Instrument	05 015 278 001	1 instrument (96 well)
LightCycler® 480 Multiwell Plate 96, white	04 729 692 001	5 × 10 plates
LightCycler® 480 Sealing Foil	04 729 757 001	50 foils
LightCycler® 480 SYBR Green I Master	04 707 516 001	5 × 1 ml (2 × conc.)
LightCycler® 480 Probes Master	04 707 494 001	5 × 1 ml (2 × conc.)
MagNA Pure LC Instrument	12 236 931 001	1 instrument
MagNA Pure LC DNA Isolation Kit I	03 003 990 001	1 kit (192 isolations)
MagNA Pure Bacteria Lysis Buffer	04 659 180 001	20 ml
Proteinase K recombinant, PRC Grade, solution	03 115 887 001 03 115 828 001 03 115 844 001	1.25 ml 5 ml 25 ml

4

4

Real-Time Multiplex PCR of Five Different DNA Targets Using the LightCycler® 480 System

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1 Introduction

One of the most interesting aspects of Real-Time PCR based on detection of fluorophoric-labeled oligonucleotides, such as hydrolysis probes, and molecular beacons, is the possibility of being able to detect conveniently multiple targets in the same PCR reaction (multiplex PCR) [1]. Ideally, a real-time multiplex PCR should be able to detect, differentiate, and provide a quantitative result for many different targets without a single target influencing the detection of one of the others (cross-talk) and without loss of sensitivity. It is evident that due to the limited number of fluorophoric labels available [2] and the significant overlap in their emission spectra, quantification of multiplex reaction products is difficult and often not possible for more than two targets [3].

Recently, Roche Applied Science launched a Real-Time PCR platform, the LightCycler® 480 System, which should be well-suited for multiplex Real-Time PCR analysis. It makes use of a broad-spectrum xenon lamp and five band pass filters for excitation of fluorophores at five different fixed wavelengths combined with six band pass filters for detection of fluorophore emission at six different fixed wavelengths. Both excitation and emission wavelengths are chosen at the peak fluorescence of commonly available fluorophores and are separated by at least 30 nm. This should allow for minimal cross-talk between the different channels and provide a platform for quantitative detection of multiple targets within the same sample.

In the study presented here, the performance of the LightCycler® 480 Instrument and accompanying software was analyzed for the multiplex detection of five different (plasmid) DNA species.

2 Materials and Methods

Five different plasmid DNAs (A-E) containing a specific amplicon sequence were constructed by standard molecular cloning techniques [4]. Hydrolysis probe-based Real-Time PCRs for each DNA species were developed with specific fluorescent-labeled probes (Table 1).

Serial dilutions of plasmid DNA were made in TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0) supplemented with 20 ng/µl of calf thymus DNA. Typical reactions contained 10 µl of 2x LightCycler® 480 Probes Master, 900 nM of primer (each), 200 nM of probe (each), and 5 µl of a plasmid DNA dilution in a total volume of 20 µl. Cycling conditions were as follows: 2 minutes at 50°C and 10 minutes at 95°C, followed by 45 cycles each consisting of 15 seconds at 95°C and 1 minute at 60°C. Data were analyzed with the LightCycler® 480 Software using the Second Derivative Maximum Method. Color compensation objects were created as described in the LightCycler® 480 Operator's Manual.

DNA	Amplicon length (kb)	Fluorophores	λ Excitation (nm)	λ Emission (nm)
A	95	Cyan 500/DB	450	500
B	105	6FAM/BBQ	483	533
C	146	VIC/NFQ	523	568
D	152	LightCycler® Red 610/BBQ	558	610
E	143	LightCycler® Red 670/BBQ	615	670
DB: Dabcyl, BBQ: BlackBerry Quencher, NFQ: non-fluorescent quencher.				

Table 1: Detection formats.

3 Results and Discussion

The dynamic range of the five different PCRs in single-target detection format (*i.e.*, one primer pair and one probe) was analyzed by a linear range of tenfold dilutions ranging from 10^6 to 10^2 copies of target plasmid in PCR. To determine the amount of cross-talk between the five different wavelength channels we determined the contribution of all fluorophores in their non-corresponding channels. After the application of color compensation as described in the LightCycler® 480 Operator's Manual, we determined that the amount of cross-talk was less than 5% in all cases (data not shown). This demonstrates that in a multiplex experiment more than 95% of the total fluorescence will be derived from the corresponding fluorophore. All five single PCRs displayed similar characteristics and showed PCR efficiencies close to 2.0. Standard deviations were very small (max. 0.56; n=4) and linearity was observed in the concentration range tested (representative examples are shown in Figure 1). On the basis of these experiments standard curves were generated by linear regression analysis.

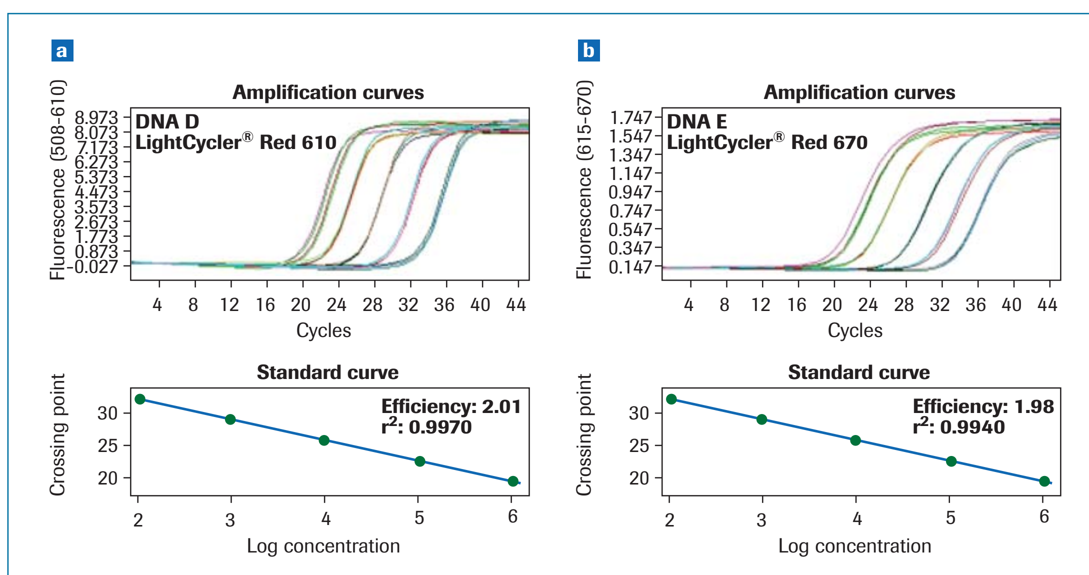

Figure 1: Examples of dynamic ranges. (a) Serial tenfold dilutions of plasmid DNAs D (LightCycler® Red 610) or **(b)** DNA E (LightCycler® Red 670) were analyzed by single-target PCR. PCR efficiencies and r^2 values are given.

Subsequently, plasmid DNAs A–E were mixed in equimolar amounts (10^4 copies/PCR). Multiplex PCR was performed in replicates of 12 and resulting mean crossing-point (Cp) values were plotted against standard curves obtained from single-target PCRs. Table 2 shows the measured Cp-values in comparison to the expected Cp-values. Measured and expected Cp-values were in accordance with each other and the largest difference was 0.6. These data show that using a pentaplex PCR and the LightCycler® 480 System, reliable detection of five different DNA targets within the same sample is possible.

	Measured [a]	Expected [b]	ΔCp
DNA A	25.7 ± 0.11	25.9	0.2
DNA B	27.0 ± 0.21	27.3	0.3
DNA C	25.9 ± 0.17	26.5	0.6
DNA D	25.3 ± 0.16	25.7	0.4
DNA E	26.0 ± 0.18	26.5	0.5

[a] Cp-values are mean of 12 replicates.
[b] Expected Cp-values were derived from standard curves.

Table 2: Measured versus expected Cp-values for DNA A-E.

4

More interesting is the investigation of the effect of differential DNA concentrations on the outcome of multiplex Real-Time PCR for each plasmid DNA. To study this, each of target DNA plasmids C, D, and E were mixed in equimolar amounts of 10^5 (sample series 1), 10^4 (sample series 2), 10^3 (sample series 3), and 10^2 (sample series 4) copies/PCR. Subsequently, target DNA A was spiked in these mixtures at final concentrations of 10^5, 10^4, 10^3, and 10^2 copies/PCR. For instance, sample 1.1 contained 10^5 copies/PCR for DNAs A, C, D and E, whereas sample 3.4 contained 10^3 copies/PCR of DNAs C, D, E and 10^2 copies/PCR of DNA A. All samples were tested in the multiplex PCR and the results are summarized in Figure 2. Cp-values for a given concentration of DNA target A did not differ significantly in the background of various concentrations of targets C, D, and E. For example, in sample 1.4, there was a low amount (10^2 copies/PCR) of DNA A present in the background of high (10^5 copies/PCR) concentrations of targets C, D and E. In this sample DNA A was detected at a Cp of ~31. In contrast, sample 4.4 contained low amounts (10^2 copies/PCR each) of all target DNAs. DNA A was detected in this sample at a Cp of ~30. For DNA targets C, D, and E the difference in Cp-value at a given concentration did not differ significantly between each one. In addition, the Cp-values for a given concentration of targets C, D, and E were similar in the background of various concentrations of DNA target A. These data demonstrate that over a range of at least a 1000-fold difference in target concentration, reliable quantification is still possible for all targets, without loss of sensitivity.

Apart from the technical specifications of the PCR apparatus, another major difference between the LightCycler® 480 Instrument and the majority of other Real-Time PCR platforms lies within the software algorithms used to convert raw fluorescence data into comprehensive results. Whereas other real-time platforms use a cycle threshold that can be arbitrarily set to a certain value by the user, the LightCycler® 480 Software calculates the second derivatives of entire amplification curves and determines where this value is at its maximum. This value (crossing point, Cp) represents the cycle at which the increase of fluorescence is highest and where the logarithmic phase of a PCR begins. By using the second-derivative algorithm, data obtained are more reliable and reproducible, even if fluorescence is relatively low. The use of this second derivative maximum algorithm might have contributed greatly to the excellent qualitative and quantitative results in the multiplex PCR described here.

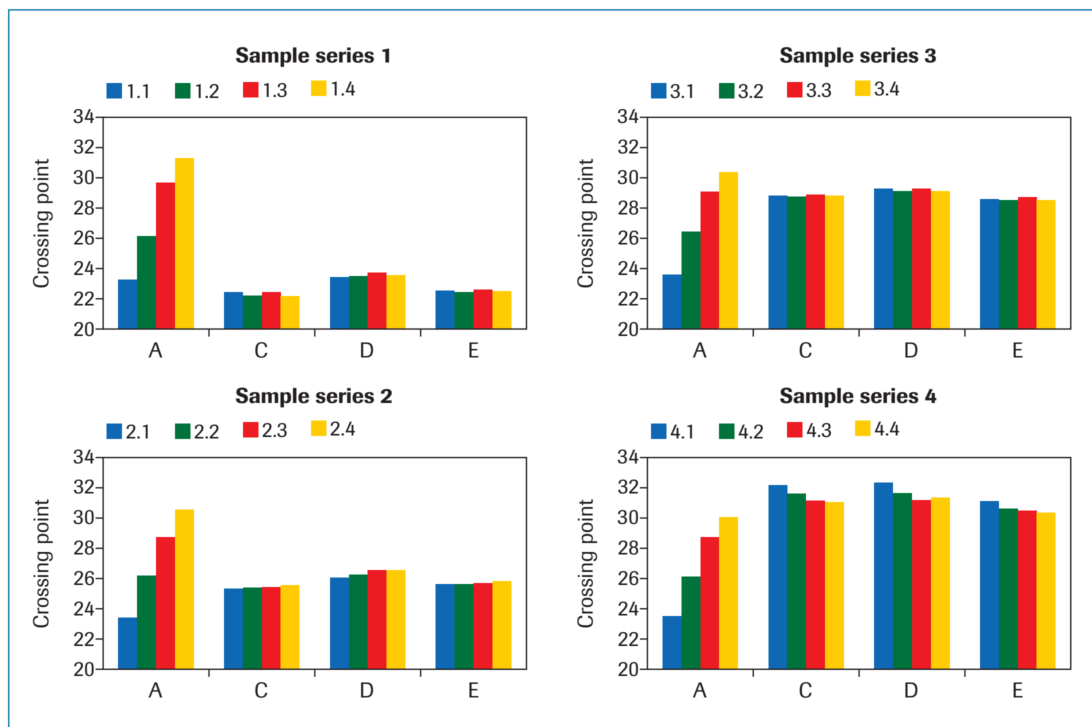

Figure 2: Effect of different target DNA concentrations in a sample on the Cp-values of the multiplex PCR.
Composition of different samples is indicated. Cp-values for each target DNA (A, C, D, and E) in a sample are displayed in the figures. All Cp-values are means of duplicates.

4 Conclusions

We made use of the excellent properties of the recently introduced LightCycler® 480 System for the reproducible detection of five different DNA targets within one reaction. It was shown that when these DNA targets were mixed, each individual DNA could still be quantitatively detected in the background of the other DNAs. In addition, a 1,000-fold difference in DNA concentration between DNAs did not significantly influence the detection of the other DNA targets. These results show that the LightCycler® 480 Instrument is a convenient and reliable platform for (quantitative) multiplex PCR detection.

5 References

1. Elnifro EM et al. (2000) Clin *Microbiol Rev* **13**:559–570
2. Lee LG (1993) *Nucleic Acids Res* **21**:3761–3766
3. Mackay IM (2004) Clin *Microbiol Infect* **10**:190–212
4. Molenkamp R et al. (2007) J *Virol Methods* **141**:205–211

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Product	Pack Size	Cat. No.
LightCycler® 480 Instrument	1 instrument (96 well)	04 640 268 001
LightCycler® 480 Multiwell Plate 96	5 × 10 plates	04 729 692 001
LightCycler® 480 Sealing Foils	50 foils	04 729 757 001
LightCycler® 480 Probes Master	5 × 1 ml (500 x 20 µl reactions) 10 × 5 ml (5,000 × 20 µl reactions) 1 × 50 ml (5,000 × 20 µl reactions)	04 707 494 001 04 887 301 001 04 902 343 001

Relative Quantification of Multiple mRNA Targets and Reference Genes in Spinocerebellar Ataxia

Michael Walter, Molecular Pathology Research and Development Laboratory, Department of Pathology, University of Tübingen, Germany.

4

1 Introduction

In the research project presented here, we studied the expression of 16 transcripts that might be predictive for the progression stage of spinocerebellar ataxia 1 and 3 (SCA1 + 3), two autosomal-dominant neurodegenerative disorders in humans (see box). We collected a total of 88 blood samples from individuals with either mild, intermediate or severe forms of ataxia. After assay design and optimization cDNAs were generated with the Transcriptor First Strand cDNA Synthesis Kit. For each individual target, the C_p values for all transcripts in each sample were determined by Real-Time PCR on the LightCycler® 480 System with Universal ProbeLibrary probes. We performed 384-well plate setup using an automated liquid handling system. Using LightCycler® 480 Software, Version 1.5, relative expression values were obtained by determining the ratio between each target and three appropriate reference transcripts, and normalized expression ratios were calculated using a common calibrator cDNA. Using this specific workflow set-up allowed generation of standard curves using five serial two-fold dilutions, which was important because several targets were known to have low expression levels. Statistical analysis was done using the LightCycler® 480 Multiple Plate Analysis Software.

A graphical overview of the entire workflow is presented in Figure 1.

BOX 1

Spinocerebellar ataxia is a group of genetic disorders, characterized mainly by progressive incoordination of gait, poor coordination of hands, speech and clumsy motion of the body due to a failure of the fine coordination of muscle movements. Generally, a person with ataxia retains full mental capacity but may progressively lose physical control. There is no known cure for spinocerebellar ataxia, and although not all types cause equally severe disability, treatments are generally focused on symptoms, not the disease itself. Both onset of initial symptoms and duration of disease can be subject to variation. The most precise means of identifying SCA, including the specific type, is through DNA analysis. The first ataxia gene was identified in 1993 for a dominantly inherited type. It was called *spinocerebellar ataxia type 1* (SCA1). Subsequently, as additional dominant genes were found, they were called SCA2, SCA3, etc. To date, at least 29 different gene mutations have been identified. Ataxias with poly CAG expansions, along with several other neurodegenerative diseases resulting from a poly CAG expansion, are referred to as polyglutamine diseases.

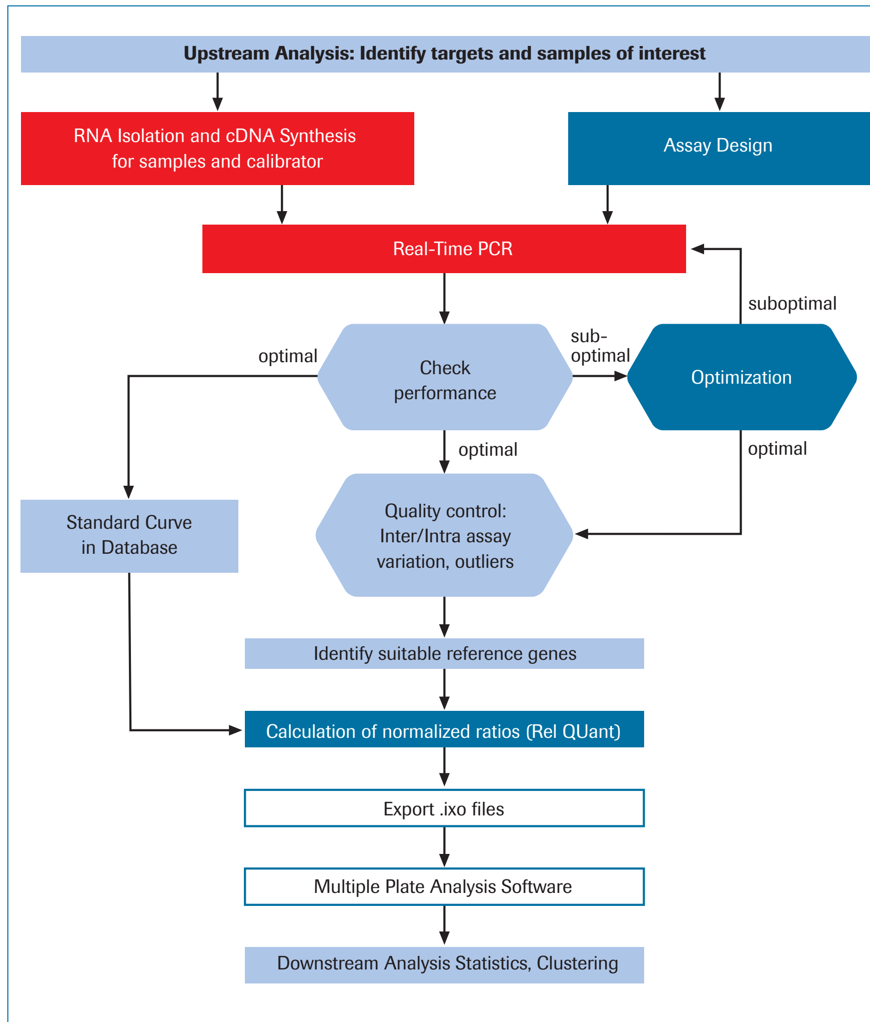

Figure 1: Schematic representation of a typical workflow for relative quantification of mRNA.

2 Assay Design

The assay design process is the most important and critical part in the workflow of a relative quantification project (shown in Figure 1). To enable high assay sensitivity and circumvent primer-dimer issues, all experiments were designed as 5' nuclease assays with Universal ProbeLibrary probes, making use of the web-based Roche assay design center. To find optimal primer-probe combinations, target sequences were uploaded to the design center[1]. Where applicable, the Ensembl transcript ID was used to identify the target and automatically retrieve the sequence from the Ensembl database[2]. Where possible, the assays were designed with intron-spanning primer pairs or probes (see Table 1). In cases where the assay design center proposed several different possible primers or probes, the top ranking assay was chosen for initial evaluation.

[1] https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp
[2] www.ensembl.org

3 RNA Extraction and cDNA Synthesis

Research blood samples from patients were collected using PAXgene vacutainer (BD Biosciences). RNA was isolated using the PAXgene RNA Isolation Kit (BD Biosciences) according to the manufacturer's recommendation. Quantification was done spectrophotometrically on a Nanodrop (Thermo Scientific) and quality was checked on a Bioanalyzer 2100 (Agilent). All RNAs had RNA integrity numbers (RIN) greater than 8 indicating good, and equally important, comparable RNA quality across the entire sample population.

Since a total of 21 different assays was performed for each of the original samples, we used a two-step RT-PCR approach for cDNA synthesis. Reverse transcription of 1 µg of total RNA was performed with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) using a combination of anchored-oligo(dT) primer, binding at the beginning of the poly A tail, and random hexamer primer according to the protocol below. To enable detection when probe binding sites reside several kb upstream of the poly-A tail, we used a broader range of primer types (use of oligo-dT primers alone can often lead to a dramatic increase in Cp values, because oligo-dT can bind somewhere in the often long poly A tail; unpublished observation). We performed one cDNA synthesis reaction per sample and 30 reactions for the calibrator cDNA. cDNAs were stored undiluted at -20°C. To prevent multiple freeze-thaw cycler, the calibrator cDNA was stored in aliquots.

cDNA synthesis protocol:

1 µg	total RNA
1 µl	anchored oligo-dT primer
2 µl	random hexamer primer
ad 13 µl	PCR-grade water
10 min	65°C, then on ice

add[3]:	4 µl	RT buffer
	2 µl	dNTP Mix
	0.5 µl	Protector RNase Inhibitor
	0.5 µl	Transcriptor Reverse Transcriptase
	10 min	25°C
	60 min	50°C
	5 min	85°C → store at –20°C

[3] Prepare as master mix and add 7 µl per reaction

4 Assay Optimization and Evaluation

Initially, all assays were tested under standard conditions, *i.e.*, using 300 nM of each primer and probe and a standard PCR protocol (see Fig. 3). To establish the standard curves, a rather narrow range of dilutions (five serial two-fold dilutions of a pool of sample cDNAs) was chosen, because several targets were known to have low expression levels. We expected and found the observed fold changes in the study described here to be small, so that a narrow standard curve was sufficient to cover the entire range of observed Cp values.

For assay evaluation, the following PCR performance criteria were considered:

- Amplification efficiency (must be > 1.8)
- Standard deviation of Cp values of replicate reactions < 0.3
- Smooth progression and high plateau signal to background noise ratio of amplification

Initial tests showed satisfactory performance for 10 of the 16 target assays (see Figure 2A for an example). All target and reference assays were analyzed using the same PCR cycling protocol and conditions. We analyzed the influence of primer and probe concentration on run performance of the remaining 6 assays. In some cases, increasing the concentration of the UPL probe led to a proportional increase in amplification signal in the plateau phase. This yielded an improvement on two assays with high signal-to-noise ratios (Figure 2 C, D). Since the general performance of the remaining four assays could not be improved, the second top ranking assay (as suggested by the UPL Assay Design Center) was tested and gave good results in all four cases (Figure 2 E, F). Table 1 gives an overview on all 16 assays and the run parameters used. The standard curves resulting from each of the final assays were stored as external standard curves in the Exor database and used later for efficiency correction (see below). The relative quantification analysis module of LightCycler® 480 Software, Version 1.5 can automatically load the correct standard curve for each assay, when the reactions in the run to be analyzed and the standard samples from the standard curve have the same target name assigned in their sample information.

Table 1: Primer and probe concentrations for the 16 target assays.
*Rank refers to assay list proposed by Universal ProbeLibrary Assay Design Center.

Gene Symbol	Ensembl	Genbank	UPL	Primer [nmol]	Probe [nmol]	Temp [°C]	Time [s]	Volume [µl]	Rank*	Efficiency
230585_at	---	AI632692	67	300	100	60	60	10	1	1.994
232939_at	---	AU152763	11	300	100				1	2.075
244218_at	---	AI374686	69	300	100				1	1.905
BANK1	ENST00000322953	AA811540	31	300	200				1	2.013
FAM3C	ENST00000359943	NM_014888	2	300	100				1	1.924
FCRL5	ENST00000368189	AW241983	21	300	100				2	1.971
FLJ32866	---	AK057428	37	300	100				1	1.973
FLJ36550	---	AW270105	60	300	100				1	1.931
IL18RAP	ENST00000264260	NM_003853	1	300	100				2	2.037
LOC285463	---	BF984434	44	300	100				1	1.945
MRPL13	ENST00000306185	NM_014078	59	300	100				2	1.853
MS4A1	ENST00000389941	BC002807	2	300	200				1	1.902
PADI2	ENST00000375486	AL049569	11	300	100				1	1.963
SIGLEC5	ENST00000222107	NM_003830	9	300	100				1	1.863
THAP6	ENST00000311638	BF685315	76	300	100				1	1.961
TSPAN13	ENST00000262067	NM_014399	67	300	100				2	2.050

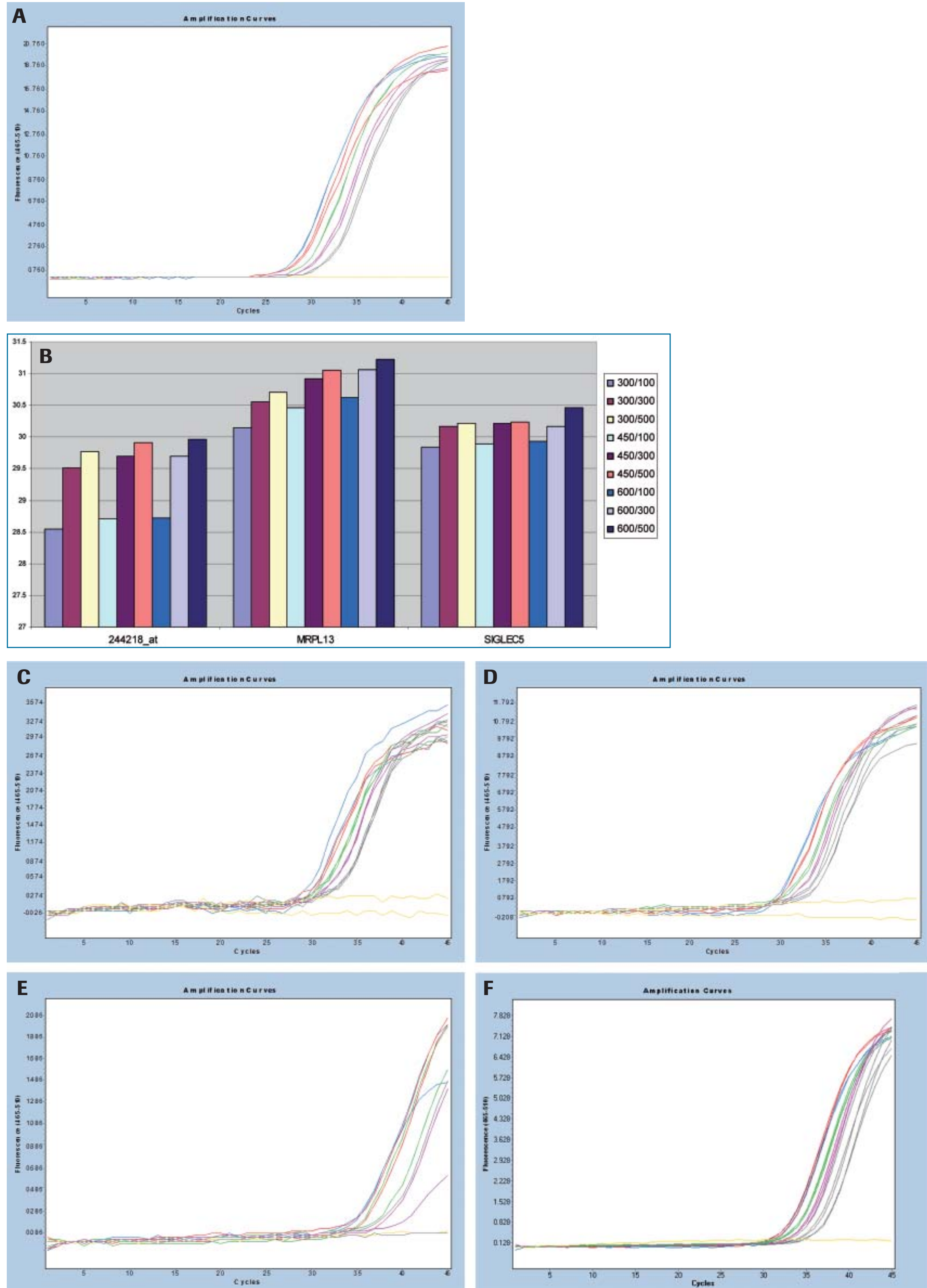

Figure 2: A) Standard curve for LOC285463, meeting quality criteria without further optimization. B) Influence of primer and probe concentration on Cp values. Three different amounts of primer were used (300, 450, 600 nmol) in combination with three different amounts of probe (100, 300, 500 nmol). Higher probe concentrations gave higher fluorescence signals but also higher Cp values. **C**), **D**) Standard curves for MS4A1 with 100 nM (C) and 200 nM (D) Universal ProbeLibrary probes. Please note the different scaling of the y axis. **E**), **F**) Standardcurves for top ranking assay (**E**) and first runner up assay (**F**) for TSPAN13.

Reference Gene Assays

Relative quantification of mRNA requires the use of one or several reference genes. For simple experimental systems, *e.g.*, treatment of cultured cells with different stimuli, a single reference gene might be suitable for normalization. For more complex sample sources, especially human sample material with high degree of genetic heterogeneity, a single reference gene whose expression is uniform throughout the entire sample population, is generally difficult to find. Thus, a combination of several reference genes must be used for normalization. When a *priori* information on reference genes suitable for a given experimental system is not available, it is highly recommended to measure several reference genes and use the most suitable one(s) for later data analysis. In our case, eight different reference gene assays available in a pre-designed and validated form from Roche Applied Science[4] were tested and could be carried out without further optimization. Five out of these eight (ACTB, G6DH, GAPDH, HPRT, PPIA), spanning the same range of Cp values as the target assays (Cps of approx. 25 to 36) were chosen, and used for all samples. Standard curves for the reference genes were also established and stored.

Study Layout and Plate Preparation

In principal, there are two ways to set up large qPCR studies. One can either do *sample maximization*, *i.e.*, analyze as many samples per plate as possible for a given assay (primer/probe combination), or, alternatively, for *gene maximization*, fill the plate with as many assays as possible for a given sample. Ideally, there is only a single plate containing all reactions for an assay, since no correction for inter-run variability is then required. This is definitely the best choice for studies where all samples can be collected and analyzed in a single batch. The set of transcripts examined in the study presented here was selected to give information on the progression of a disorder. For each individuum investigated, the same set of transcripts can be expected to be analyzed over a long period, initially and later on. Therefore, we decided to choose the gene maximization approach and analyzed all assays for a given sample in a single run on the same plate. To enable comparison of results between different plates, a common cDNA (calibrator) was included in each plate.

Figure 3 shows the layout for the 384-well plates used for qRT-PCR. This experimental design allowed us to analyze four samples plus the common calibrator and negative controls per plate. Twenty-two 384-well plates were required in total to complete the measurement of the 88 samples in this study.

Plates were set up with the aid of an automated liquid handler (Biorobot Universal Platform, Qiagen). For each plate, five master mixes were prepared containing the cDNA for four samples and the calibrator, respectively. A sixth master mix did not contain any template and served as negative control (see tables below). Eight microliters of master mix were dispensed in each well of the 384-well plate, and 2 µl of primer-probe mix presented in a V-bottom 96-well plate (Sarstedt) was added to the reaction mix (see below). PCR reactions for sample and calibrator cDNA were prepared in triplicates, while no-template control reactions were run only once per plate. The plate was centrifuged 2 min at 1,500 × g prior to PCR amplification.

Master mix for samples/calibrator

346.5 µl	LightCycler® 480 Probes Master
13.9 µl	cDNA
221.8 µl	PCR-grade water
8 µl/well	+ 2 µl primer probe mix

Master mix for no template control (NTC)

132 µl	LightCycler® 480 Probes Master
79.2 µl	PCR-grade water
8 µl/well	+ 2 µl primer probe mix

Primer-probe mix for target assays:

15 µl	5 µM forward primer
15 µl	5 µM reverse primer
5 µl	UPL probe
65 µl	PCR-grade water

Primer-probe mix for reference assays:

10 µl	Primer (premixed)
10 µl	UPL probe
80 µl	PCR-grade water

[4] https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=070000

LightCycler® 480 program for amplification and detection of PCR products

Program Name	Denaturation		
Cycles	1	Analysis Mode	None

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:10:00	4,80		0	0	0

Program Name	Amplification		
Cycles	45	Analysis Mode	Quantification

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:00:10	4,80		0	0	0
60	Single	00:01:00	2,50		0	0	0

Program Name	Cooling		
Cycles	1	Analysis Mode	None

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
40	None	00:00:05	2,50		0	0	0

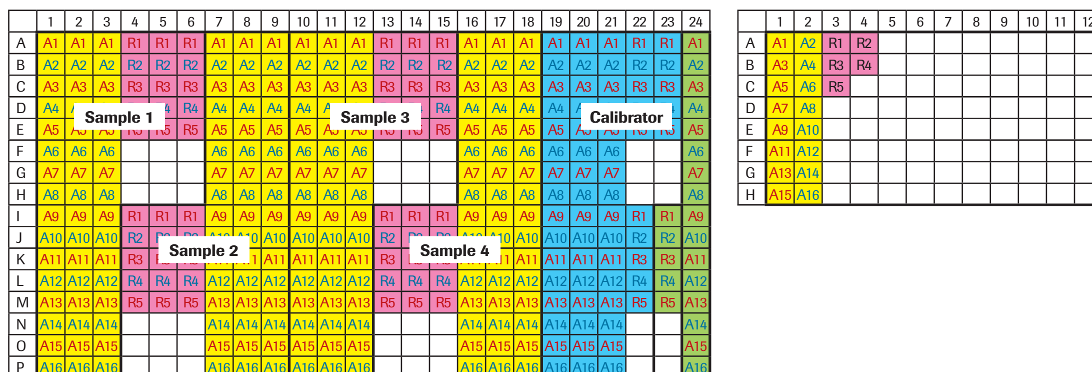

Figure 3: Layout of white LightCycler® 480 384-well plates (left) and LightCycler® 480 96-well V-bottom plates containing primer probe mixes (right). A1-A16: target gene assays 1 to 16, R1-R5: reference gene assays 1 to 5. Position of sample cDNA is colored yellow and pink for target and reference gene assay, respectively. Positions of calibrator cDNA and NTCs are depicted in blue and green, respectively.

5 Quality Control: Outlier Removal, Intra- and Inter-Assay Variability

After completion of each run, a visual inspection of the resulting amplification curves was done and the Cp standard deviations were evaluated. A result was called an outlier when the Cp standard deviation was larger than 0.3 and a single reaction displayed a clearly different amplification curve. All together 111 reactions (1.3%) were excluded from further analysis.

For the second quality control step, we calculated the Cp values´ coefficient of variation (%CV) for the replicates of each sample to determine the intra-assay variability. The %CV values for each assay are displayed as box plots (Figure 4). Intra-assay variability was very low for all assays, with at least 75% of all triplicate measurements having a CV of less than 1%.

Since the calibrator cDNA was included in each 384-well plate run, the inter-assay variability could be calculated in a similar way. These values were also found to be very small (data not shown).

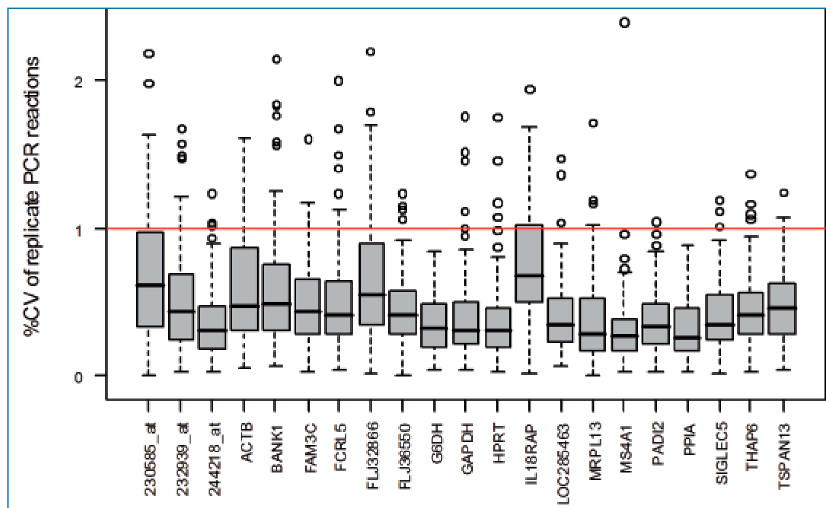

Figure 4: CV (coefficient of variation) values for PCR replicates, represented as box plots for all 21 assays (16 targets, 5 reference genes) included in out study. The horizontal bars indicate the median, the box spans the inter quartile range (25th - 75th percentile). Outliers are depicted as open circles; the red horizontal line marks 1% CV.

4

6 Selection of Reference Genes

The RNAs analyzed in this study were obtained from individuals affected by different progression states of either the SCA1 or SCA3 disorder. Since the variability of expression levels in human research samples can be higher than for cell culture systems or animal models, we used three reference genes for normalization of the expression ratios. Since *a priori* knowledge of suitable reference genes for SCA patients was not available, we measured the expression of five different reference genes in the sample population using the geNorm approach (Vandesompele et al., 2002) to define the three most stably expressed reference genes. With this method, the pair-wise ratios between all reference genes were calculated for all samples. Stably expressed reference genes will show little variation in different samples, while transcripts whose expression changes between samples will show large variation in the ratios. Using this approach, a stability measure (M) is calculated, indicating whether a transcript shows high degree of variation or not (see Vandesompele et al. (2002) for details on the method).

Table 2 shows an overview of the M values and %CV of all reference gene assays. GAPDH and PPIA showed the highest degree of variation and were thus not used as reference genes, while ACTB, HPRT and G6DH were used subsequently for normalization.

	CV	M (geNorm)
ACBT	40.61%	1.1096
G6DH	36.94%	1.0530
GAPDH	58.86%	1.4255
HPRT	50.50%	1.1112
PPIA	70.68%	1.4686
Mean	51.52%	1.2336

	CV	M (geNorm)
ACBT	35.36%	0.9467
G6DH	34.61%	0.8769
HPRT	46.66%	0.9860
Mean	38.88%	0.9365

Table 2: M-values and coefficient of variation of all reference genes (left) and after removal of the two most unstable transcripts (right).

7 Relative Quantification Analysis

After identification of the three most suitable reference genes, target-to-reference ratios were calculated using the relative quantification analysis module of the LightCycler® 480 Software, Version 1.5. Each sample on a 384-well plate was assigned as either target or reference, cDNA samples were named unknown, the wells containing the calibrator serves as positive control/calibrator (Figure 5). Based on this classification, the appropriate target name for each assay was entered in the LightCycler® 480 Software, Version 1.5 sample editor. To allow for efficiency correction during the calculation of expression ratios, the standard curves resulting from the assay evaluation step were stored in the Exor database using the same target name. This matching of target names allowed the software to automatically detect and extract the standard curve data and use the corrected efficiency value in the expression ratio calculation. Prior to calculation of target/reference ratios, all initially identified outlier reactions were excluded from the underlying absolute quantification analyses. The parameters selected in the relative quantification software dialog box are shown in the right part of Figure 5.

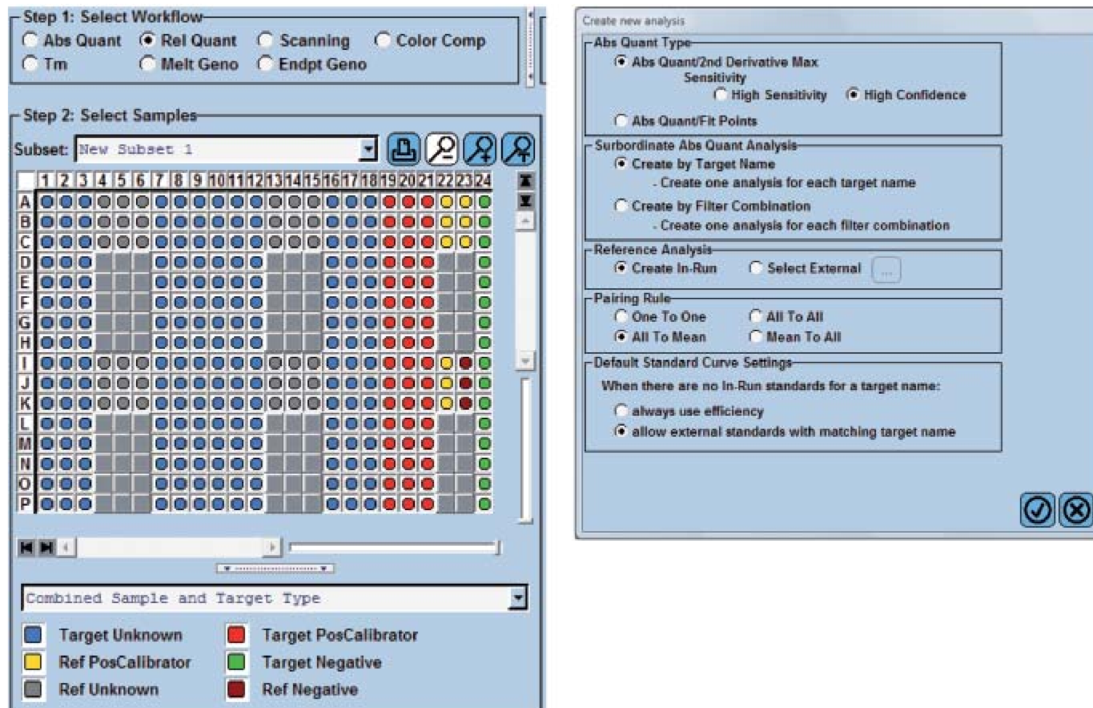

Figure 5: Advanced relative quantification:
Left panel: Assignment of sample attributes in sample editor window. Right panel: RelQuant analysis dialog window.

4

8 Downstream Multiple Plate Analysis and Statistic Calculations

The entire data set for this study corresponded to twenty-one 384-well plates. The LightCycler® 480 Multiple Plate Analysis Software was used to easily compile these multiple-run data, extract statistic paramenters and thus obtain biologically meaningful information.

Files containing the analysis results from each individual experimental run were exported from the LightCycler® 480 database as .ixo files (individually or all together in one batch) and then imported into the LightCycler® 480 Multiple Plate Analysis Software. This resulted in a new database object, holding all ixo-files belonging to the same study in a common *study object*, thus making any reformatting steps and error-prone copy-paste operations unnecessary.

To enable subsequent data compilation and analysis, so-called properties were defined: *conditions* refers to SCA1 or SCA3; *stage* refers to mild or intermediate or severe forms of ataxia. To analyze each sample set, corresponding values for each property were assigned to each sample and samples were grouped according to type of disorder (SCA1 versus SCA3), and progression category (*mild, intermediate* and *severe*). As summarized in Fig. 6, this procedure led to data compilation resulting in a single .xls file holding all the data. Tables included in this file show the normalized ratios with standard deviations for all assays and all samples together with their properties. The provided workflow option *RelQuant Summary* was chosen to display normalized ratios.

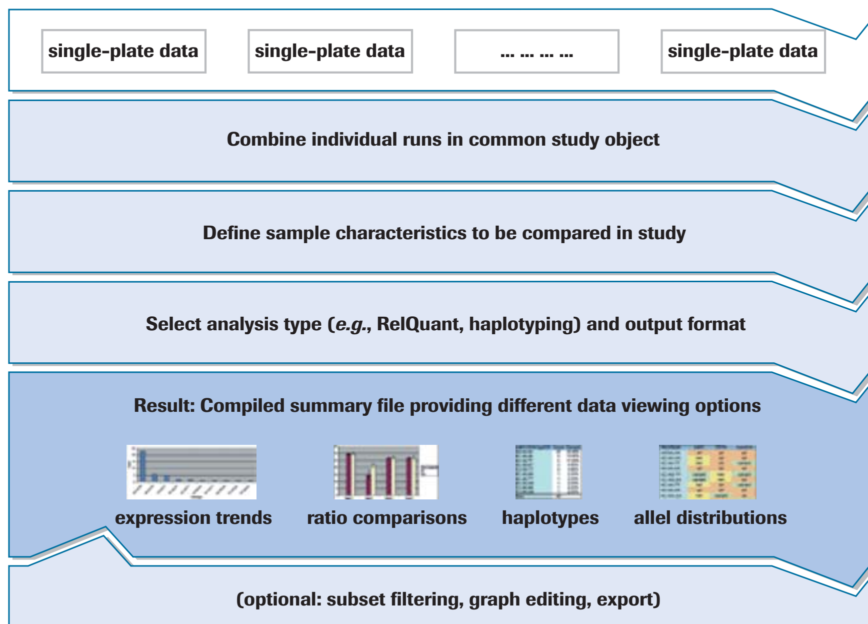

Figure 6: LightCycler® 480 Multiple Plate Analysis Software Workflow

Several advanced options were used to inspect and visualize the target assays individually or in groups in different ways. For instance, the LightCycler® 480 Multiple Plate Analysis Software allows assay distributions to be displayed as box-and-whisker plots. It also allows diagrams to be filtered for any property/value combination, which in turn allows an easy inspection of the range and variation of ratios within the biological replicates, such as type or stage of disease (see Figure 7A, B). Any filtering applied in an analysis worksheet is applied to all worksheets and all charts are automatically updated. An alternative way to see the distribution of the ratios for individual assays is to display the results as signal histogram. Up to four assays can be displayed in parallel. Additionally, the individual ratios for each sample can be shown as bar plots below the histogram (see Figure 7C, D).

The most common question addressed when performing Real-Time PCR experiments is whether the expression of a transcript differs as a function of two or more different physiological conditions. This kind of question is addressed in the criteria comparison worksheet provided by the LightCycler® 480 Multiple Plate Analysis Software (see Figure 7E, F). For each assay, the average ratio plus standard deviation for each value of the selected property is calculated and displayed in tabular form. These values are used to graphically display the results in a bar plot. If more than one property is assigned to the data, then an additional filter can be applied to show the results for each subordinate property separately (see Figure 7E, F). To check if the observed differences are by chance alone, a two-tailed Student's t-test is performed for each combination of values for the selected property based on the filter combination in the criteria comparison (data not shown). For a more complex statistical analysis, the calculated average and standard deviation or the summary table containing the normalized ratios can be easily exported and transferred to more specialized software.

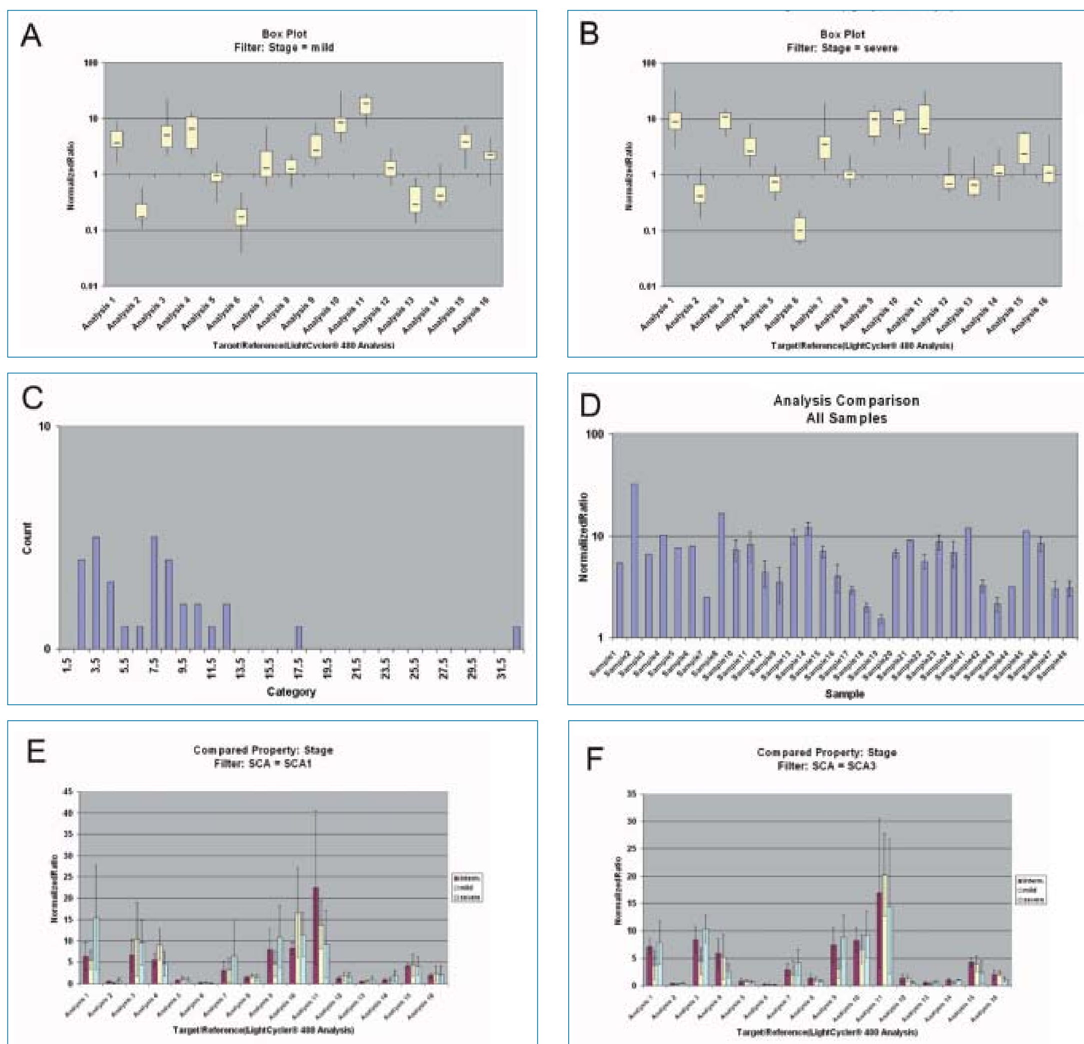

Figure 7: Data evaluation and analysis in Multiple Plate Analysis Software output. A, **B**: Box-and-whisker plots of normalized ratios for *mild* and *severe* stage patients for 16 targets assays analysed. The horizontal bars indicate the median, the box spans the inter quartile ratios for individuals and the *whiskers* delineate the minimum and maximum of all data. **C**) Histogram for a single selected assay with bin width 1 showing a right tailed distribution of a data, which are displayed in **D**) for all samples individually. **E**, **F**) Average ratios and standard deviations for all values within the selected property *Stage*. Additional filtering for the two different diseases was applied (**E**: SCA1, **F**: SCA 3).

9 Conclusion

The aim of the present study was to investigate how the up- and downregulation of certain cellular transcripts is related to the type and severity of spinocerebellar disorders, SCA1 and SCA3. Primers and probes specific for targets that had been previouly identified as candidate markers on array platforms were easily obtained using the free, web-based Roche Universal ProbeLibrary Assay Design Center (www.universalprobelibrary.com). Combining the Transcriptor First Strand cDNA Synthesis Kit, Universal ProbeLibrary probes and the LightCycler® 480 Instrument in a smooth workflow easily produced high-quality two-fold dilution standard curves crucial for the detection of transcripts expressed at low levels. After rapid Real-Time PCR, the LightCycler® 480 Multiple Plate Analysis Software provided easy access for the analysis and direct comparison of different progression states of ataxia. Large amounts of high-quality, biologically relevant data were generated in a short period of time.

In summary, the LightCycler® 480 System in combination with the Transcriptor First Strand cDNA Synthesis Kit and Universal ProbeLibrary assays, provides a straightforward and productive workflow for processing large numbers of samples for relative quantification using high-throughput Real-Time PCR.

10 References

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Ordering Information

Product	Cat. No.	Pack Size
Transcriptor First Strand cDNA Synthesis Kit[1)]	04 379 012 001 04 896 866 001 04 897 030 001	1 Kit (50 reactions) 1 Kit (100 reactions) 1 Kit (200 reactions)
LightCylcer®480 Instrument II, 96-well	05 015 278 001	1 Instrument[2)]
LightCylcer®480 Instrument II, 384-well	05 015 243 001	1 Instrument[2)]
LightCylcer®480 Software Version 1.5	04 994 884 001	1 Software Package
LightCylcer®480 Multiple Plate Analysis Software	05 075 122 001	1 Software Package
LightCylcer®480 Multiwell Plate 96, white	04 729 692 001	50 Plates / 50 Foils
LightCylcer®480 Multiwell Plate 384, white	04 729 749 001	50 Plates / 50 Foils
LightCylcer®480 Multiwell Plate 96, clear	05 102 413 001	50 Plates / 50 Foils
LightCylcer®480 Multiwell Plate 384, clear	05 102 430 001	50 Plates / 50 Foils
LightCylcer®480 Probes Master (2× concentrated)	04 707 494 001 04 887 301 001 04 902 343 001	5 × 1 ml (500 × 20 µl reactions) 10 × 5 ml (5000 × 20 µl reactions) 1 × 50 ml (5000 × 20 µl reactions)
Universal ProbeLibrary Set, Human	04 683 633 001	1 Set[3)]
Universal ProbeLibrary Set, Mouse	04 683 641 001	1 Set[3)]
Universal ProbeLibrary Set, Rat	04 683 650 001	1 Set[3)]
Universal ProbeLibrary Extension Set	04 869 877 001	1 Set[3)]
Universal ProbeLibrary, Human PBGD Gene Assay	05 046 149 001	500 reactions
Universal ProbeLibrary, Human HPRT Gene Assay	05 046 157 001	500 reactions
Universal ProbeLibrary, Human ACTB Gene Assay	05 046 165 001	500 reactions
Universal ProbeLibrary, Human PGK1 Gene Assay	05 046 173 001	500 reactions

[1] For detailed information, visit www.roche-applied-science.com/pcr

[2] Instrument package includes LightCycler® 480 Instrument, LightCycler® 480 thermal block cycler unit (96- or 384-well), LightCycler® 480 software, LightCycler® 480 Instrument Operator's Manual, LightCycler® 480 Xenon Lamp (spare lamp). A Pentium desktop PC is supplied with the instrument.

[3] For detailed information, visit www.universalprobelibrary.com

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4

Mutation Scanning Using High Resolution Melting or dHPLC: A Performance Comparison Study

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1 Introduction

High Resolution Melting (HRM) is a novel and promising screening technique used for mutation analysis and detection. Its application prevents the need to sequence all the exons of a gene when looking for novel mutations. The method is therefore time- and cost-effective for the analysis of large genes comprising many exons.

In the research project described here, we compared HRM on the LightCycler® 480 System to denaturing high-performance liquid chromatography (dHPLC), a more conventional method for mutation scanning. A WAVE 4500 HT System (Transgenomic Inc.) has been used for many years in our accredited human genetic laboratory for screening human samples by dHPLC, to identify unknown mutations that can then be characterized in detail by sequence analysis.

We asked whether it was possible to transfer dHPLC assays to the HRM method, with as few changes as possible in primer sequence or assay optimization. The establishment of assays and testing involved 12 exons of the human *BRCA1* gene, linked to breast and ovarian cancer, in previously characterized research samples, and and – if available – plasmids serving as controls. These samples were analyzed in parallel, using both the WAVE and the LightCycler® 480 Systems. Results obtained with these methods were compared to each other in terms of sensitivity and specificity.

2 Materials and Methods

Template preparation

Genomic DNA was prepared using the MagNA Pure LC System (Roche Applied Science) from 200 µl whole blood per sample.

PCR setup

Based on previously established PCR conditions, the first step was to determine the optimum annealing temperatures and Mg^{2+} concentrations for PCR using the LightCycler® 480 High Resolution Melting Master kit. The following components and volumes were used in a total reaction volume of 20 µl each for performing PCR on the LightCycler® 480 Instrument:

Component	Volume	Final concentration
DNA or plasmid (approx. 50 ng/µl)	0.5 -1 µl	approx. 25 ng
LightCycler® 480 High Resolution Melting Master (2×)	10 µl	1× conc.
Primer F (5 µM)	0.8 µl	0.2 µM
Primer R (5 µM)	0.8 µl	0.2 µM
DMSO	Y µl	
$MgCl_2$ (25 mM)	X µl	
PCR-grade water	filled to 20 µl	
Total	20 µl	

Table 1: Components used for PCR on the LightCycler® 480 Instrument

First, the optimum Mg^{2+} concentrations for all PCR fragments of the *BRCA1* gene were established with a touchdown PCR program (annealing temperatures from +65 to +53°C). Mg^{2+} concentrations between 1.5 to 3.5 mM were evaluated, and 5% to 10% DMSO was added to some of the preparations. We used a 3-step protocol, including initial denaturation for 2 min at +94°C; 35 cycles of +94°C, 30 sec / $T°_{ann}$, 30 sec / +72°C, 1 min; final elongation at +72°C for 7 min, and finally cooling to room temperature. The final annealing temperatures used are summarized in Table 2. Primer sequences are listed in Table 3.

Gene	Exon	Size of the PCR product	Annealing temperature [°C]	Mg^{2+} [mM]
BRCA1	2	319 bp	58	2.5
	9	292 bp	58	2.5
	11A	394 bp	58	2.5
	11B	399 bp	58	2.5
	11E	445 bp	58	2.5
	11H	329 bp	56	2.5
	11I	430 bp	58	2.5
	11K	313 bp	58	2.5
	12	327 bp	54	2.5
	15	482 bp	54	2.5
	20	380 bp	53-65 (touchdown PCR)	3.0
	23	234 bp	58	2.5

Table 2: LightCycler® 480 System PCR conditions for the examined exons of the *BRCA1* gene

Exon	Forward primer	Reverse primer
9	5' - TGCCACAGTAGATGCTCAGT - 3'	5' -CACATACATCCCTGAACCTAAA - 3'
11 A	5' - TAGCCAGTTGGTTGATTTCC - 3'	5' - CCCATCTGTTATGTTGGCTC - 3'
11 E	5' - TTCAAAACGAAAGCTGAACC - 3'	5' - TTGGAAGGCTAGGATTGACA - 3'
11 H	5' -TGAACTTGATGCTCAGTATTTGC - 3'	5' - AGTCCAGTTTCGTTGCCTCT - 3'
11 K	5' - TTCCTGGAAGTAATTGTAAGCA - 3'	5' - TAACCCTGAGCCAAATGTGTAT - 3'
20	5' - TGCTAGGATTACAGGGGTGAG - 3'	5' - TTTATGTGGTTGGGATGGAAG - 3'

Table 3: Primers used for the analysis of different exons of the *BRCA1* gene.

3 Results and Discussion

For mutation analysis of the *BRCA1* gene, we had previously designed primers for amplification of 34 fragments, covering 23 exons. The resulting amplicons have been used in many routine procedures, such as PCR, dHPLC analysis and certain fragments had also been sequenced. Twelve out of these 34 PCR fragments were selected for High Resolution Melting (HRM) analysis with the LightCycler® 480 System and dHPLC in comparison. The same primers were used as previously for dHPLC and sequencing.

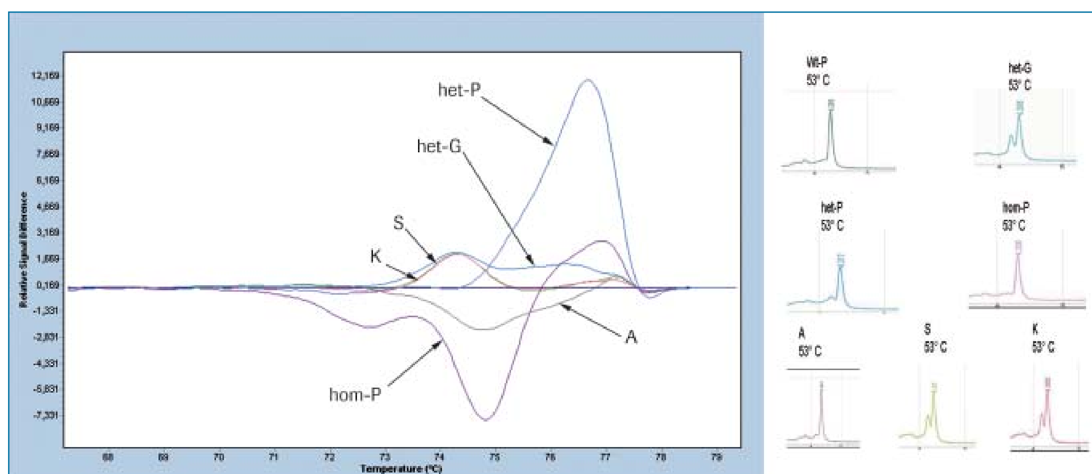
For most, but not all selected fragments, both wild-type and heterozygous or homozygous mutation controls were available. Below, for exons 9, 11 and 20, the comparative analysis obtained by dHPLC and HRM is discussed in detail for 6 out of the 12 analyzed fragments. For the analysis using the LightCycler® 480 System´s Gene Scanning Software module, the wild-type version of the plasmid was always selected as the baseline for comparison.

Exon 9:

The PCR product of exon 9 has a size of 292 bp, is rich in repeats and contains 2 known mutated sites.

Three different mutation controls were available, *i.e.*, the genomic heterozygous mutation control (het-G; mutation +64delT IVS9) inside intron 9 and one heterozygous and one homozygous mutation control as plasmid (het-P and hom-P; mutation c.676C>A). Both mutations are located near the center of the PCR fragment.

Three unknown samples A, S and K were analyzed (see Figure below). The difference between the heterozygous controls (het-P and het-G) and the wild type (Wt-P) was clearly visible both by HRM (left) and dHPLC (right). In addition, both methods provided an unambiguous result for the two samples S and K (both het-G). Sample A looked similar to hom-P in dHPLC, but different from all other samples in HRM. This finding was further confirmed by sequencing, showing that the genotype of sample A was hom-G. In addition, the HRM method allowed the demonstration of a clear difference between het-P and hom-P samples, while this small difference was not easily detected by the dHPLC method.

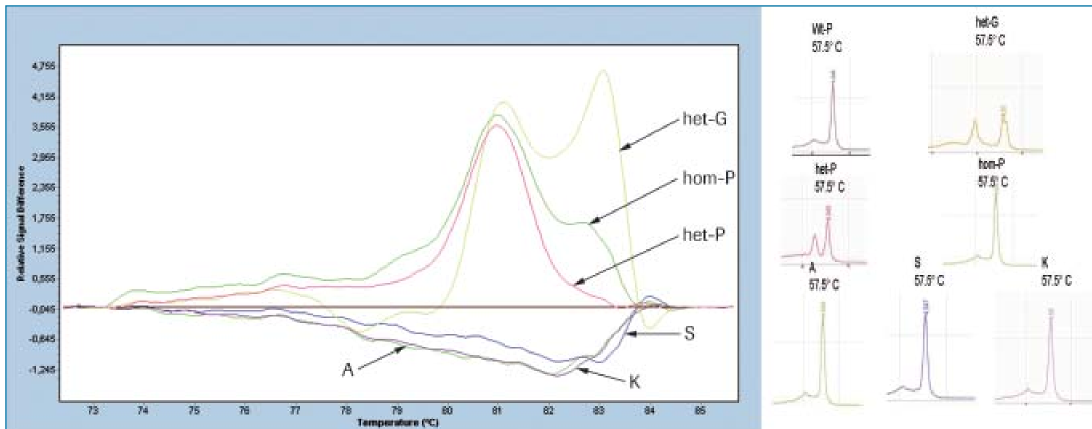


Exon 11A:

Exon 11A comprises a PCR fragment of 394 bp. It is a relatively long fragment carrying two known mutations, with many thymidine repeats in its front part.

Three mutation controls were used for this fragment, *i.e.*, the genomic heterozygous mutation control (het-G; mutation c.969_970insTCATTAC) plus one heterozygous and one homozygous control for a specific SNP (het-P and hom-P; mutation c.999A>T), both available as plasmids. Both mutations are > 50 bp away from the forward and reverse primers.

Three unknown samples A, S and K were analyzed. The two mutations het-G and het-P could be clearly differentiated by dHPLC and HRM analysis. With either method, all three unknown samples A, S and K were found to correspond to the wild type. Compared to dHPLC, the HRM analysis gave a better resolution in some cases, allowing a clear differentiation between *e.g.*, hom-P and the wild-type, Wt-P.



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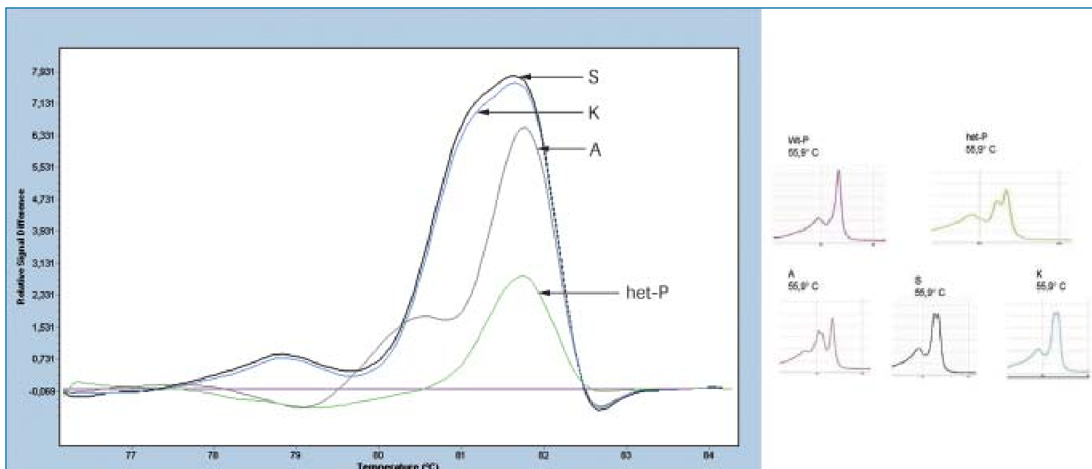
Exon 11E:

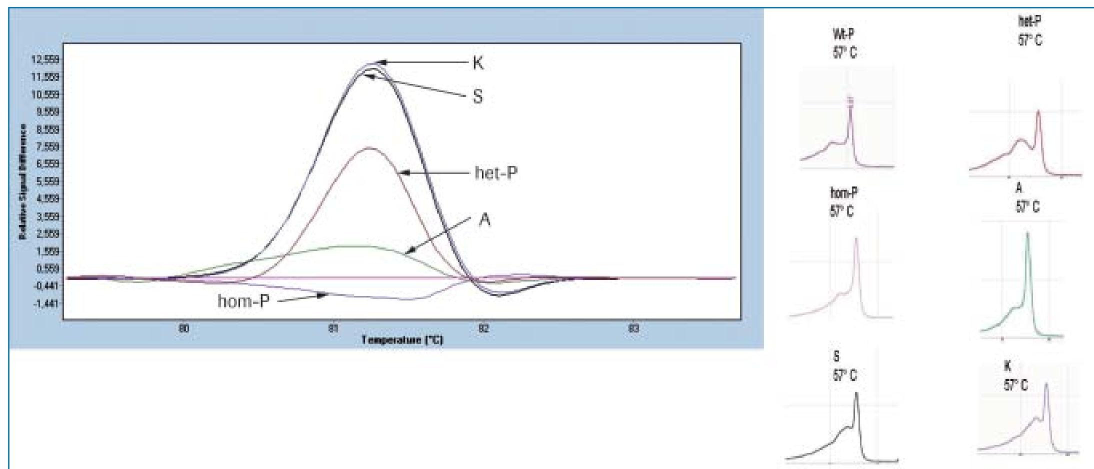
The PCR product of exon 11E has a size of 445 bp, which is relatively long both for dHPLC and HRM analysis. The control used for this examination was mutation c.2187A>T in heterozygous constellation (het-P). This mutation is located closer to the 3´ region of the PCR fragment and could be clearly differentiated both by dHPLC and HRM. Again, the three samples A, S and K were examined for mutations in this exon. Both methods allowed to detect the difference between the samples S and K on the one hand and sample A on the other hand. Subsequent sequencing demonstrated that S and K carry mutation c.2196G>A in heterozygous constellations and A carries another mutation c.2201C>T in a heterozygous constellation. Both systems, HRM and dHPLC, identified this difference clearly.



Exon 11H:

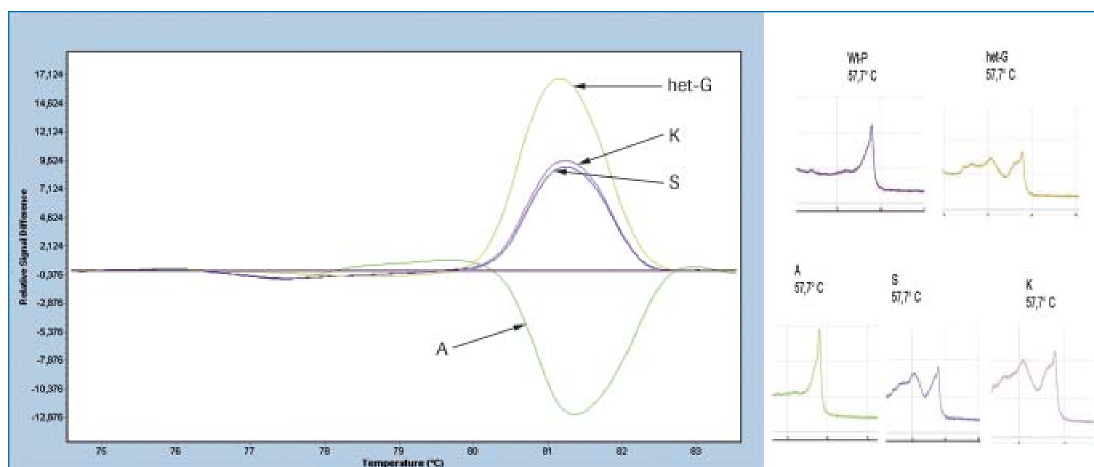
The PCR product of exon 11H has a size of 329 bp and a low GC content of 40%. Due to its relatively high adenine content, the identification of this product by dHPLC system is relatively difficult. The mutation control available for this exon was mutation c.2765T>A in homozygous and heterozygous constellations (hom-P and het-P). Both dHPLC and HRM allowed the detection of this mutation in heterozygous constellation compared to the wild type, Wt-P. However, neither HRM nor dHPLC were able to clearly differentiate hom-P from wild-type controls.

The three samples A, S and K were examined. The two samples S and K carry mutation c.2731C>T in heterozygous constellation. This difference could be demonstrated both by the dHPLC and LightCycler® 480 System and was verified by sequencing. Sample A was found in the same group as hom-P and het-P: It could be concluded that it is different form the wildtype and samples S, K, but only sequencing would be able to characterize it completely.



Exon 11K:

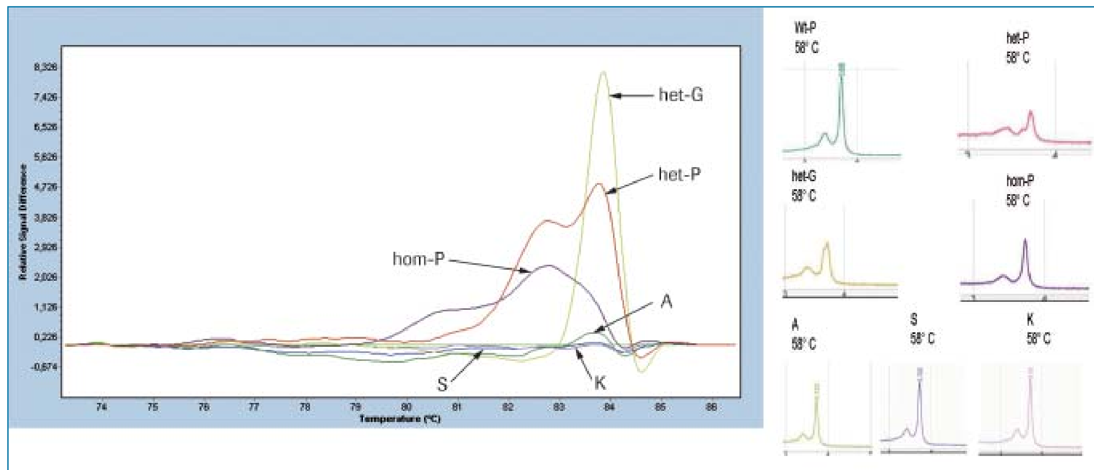
The PCR product of exon 11K has a size of 313 bp. Mutation c.3667A>G in heterozygous constellation was analyzed (het-G) and samples A, S and K were examined. Samples S and K, both carrying mutation c.3667A>G in heterozygous constellation, could by clearly identified both with dHPLC and on the LightCycler® 480 System. In addition, the LightCycler® 480 System allowed to detect the difference between sample A carrying mutation c.3667A>G in homozygous constellation, and the wild-type. This difference was much more difficult to see using dHPLC.



Exon 20:

The PCR product of exon 20 has a size of 380 bp. For this exon, the genomic mutation control c.5382_5383insC in heterozygous constellation and the plasmid mutation controls c.5350G>C were examined in heterozygous and homozygous constellations.

The samples A, S and K were examined. Both dHPLC and HRM allowed differentiation of the heterozygous genomic mutation control (het-G; c.5382_5383insC) and the plasmid mutation control (het-P; c.5350G>C) in the heterozygous constellation. The plasmid mutation control c.5350G>C in homozygous constellation (hom-P) could be only clearly differentiated from the wild-type via HRM analysis. The three samples A, S and K demonstrated no mutation and were therefore identified as wild type wit both methods. In total, the result obtained for this exon was very clear.



4 Discussion and Conclusion

Using the *BRCA1* gene as a study model, it could be demonstrated by comparative analysis of two gene scanning methods, the more traditional dHPLCS and the more recently developed High Resolution Melting, that both systems are highly comparable with respect to sensitivity and specificity.

However, the comparative analysis performed in our laboratory also demonstrated that the LightCycler® 480 System is much more convenient to use in comparison to the dHPLC method, since the HRM method is homogeneous and both the PCR and subsequent analysis take place in the same instrument with no need to open the reaction tubes. A further benefit of the LightCycler® 480 System is the option to use both plasmids and genomic DNA as target material, thus permitting the addition of wild-type material immediately prior to the PCR.

Subsequent sequencing of the PCR product following enzymatic or column purification can also be performed without problems, due to the special properties of the DNA-binding dye in the LightCycler® 480 High Resolution Melting Master, which is quite different from SYBR Green I in this regard. Although the dHPLC method allows both the use of plasmids and genomic DNA as target material, wild-type material is normally added to the reaction mixture after the PCR (requiring the tubes to be opened), because this approach significantly improves the dHPLC results. However, this means that an additional labor-intensive work step must be carried out, with the inherent risk of contamination.

Another benefit when performing HRM using the LightCycler® 480 System compared to dHPLC is the much reduced need for servicing and maintenance. High performance liquid chromatography instruments in general require many more service calls than a plate PCR thermocycler. In addition, the daily handling and regular maintenance procedures of the dHPLC system are much more complex and time-consuming.

We observed that HRM analysis can be more difficult in some cases when the mutations are in proximity (≤16 bp away) to one of the PCR primers. This phenomenon did not occur to the same extent for analyses by dHPLC. When transferring assays from the dHPLC to the HRM method, redesign of some of the primers may therefore be required in certain cases. Bearing in the mind that in our case we did not carry out any primer redesigns, the number of assays that were successfully transferred without any optimization was remarkably high. Even in cases where primer dimers were present (as detected by classical melting curve analysis; data not shown), the data could be analyzed satisfactorily. Although plasmids were not available as controls for all exons and mutations, samples with new, unexpected variants (*e.g.*, exon 9 in sample A) could be amplified and easily characterized further by sequencing.

Neither HRM nor dHPLC analysis permitted reliable conclusions with regard to the exact genotype of a fragment based on the melting or migration pattern of a mutated fragment alone. To fully characterize samples differing from the wild-type controls, we therefore always included a sequencing step. This applied to results for both the HRM and the dHPLC method.

With respect to the analysis of the heterozygous constellation of mutations, both systems demonstrated unambiguous results. However, analysis with the LightCycler® 480 System also allowed us to detect a difference between the heterozygous and the homozygous constellation of mutations (see *e.g.*, *BRCA1* exon 11K, exon 20). Although this differentiation can be achieved with the LightCycler® 480 System, it is still strongly recommended to complete the analysis by analyzing the homozygous mutation control in comparison to wild-type, because the recognition of the mutation in the homozygous constellation strongly depends on fragment-specific factors, such as amplicon size and GC content.

In summary, the transfer of gene scanning protocols from well established PCR/dHPLC-based protocols to HRM on the LightCycler® 480 System proved to be straightforward, allowing us to achieve very clear results for PCR fragments having a medium size of ≤500 bp with a moderate GC content. This means that very little effort is required to replace dHPLC Systems with the LightCycler® 480 System for mutation scanning and detection in biomedical research and testing.

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Endpoint Genotyping to Study Associations of Cannabinoid Receptor Variants with Adiposity

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1 Scientific Background Information

The epidemic proportions of obesity have generated a lot of research into the factors underlying this complex disease as well as to mechanisms linking its metabolic and cardiovascular complications (1). Recent data suggest that the endocannabinoid system is a key circuit contributing to central appetitive regulation and that its abberant activation may contribute to obesity (2). These findings have been corroborated by pharmacological studies where the cannabinoid receptor (CB1) has emerged as a suitable target for the treatment of obesity (3).

2 Aim of the Study

We aimed at evaluating, by means of a genetic association study, whether genetic variation at the cannabinoid receptor locus (CNR1) could have an effect on adiposity and fat distribution. The synonymous G/A single nucleotide polymorphism (SNP) at position 1422 of the CNR1 gene was selected because it resides in the gene's coding region (threonine at position 453) and because it is a common polymorphism with a minor allele frequency of 0.258 (HapMap reference CEU panel [4]).

3 Materials and Methods

1064 obese subjects were recruited from individuals consulting a local obesity clinic. 251 healthy control individuals were enrolled as well.

G1422A (rs1049353) genotypes were determined by a commercial hydrolysis-probe based genotyping assay on the LightCycler® 480 System (Fig. 1). Blank samples and samples with known genotype were included as negative and positive controls. We achieved 100% concordance in the analysis of duplicate samples.

Statistical analyses were performed using SPSS software, version 12.0 (SPSS, Chicago, IL, USA). Linear regression was used to adjust anthropometric parameters for age and BMI.

For the analysis of population stratification, 170 lean controls were matched on age and gender with 170 obese individuals by the Match utility (5) and analyzed for ten TaqMan® SNP genotyping assays (ABI, Foster City, USA) selected from the marker set used by Yoon et al. to estimate population structure (6). All TaqMan® assays were run as 5µl reactions in 384-well format on the LightCycler® 480 System using a standard protocol. Genotype data were analyzed with the Multi Plate Analysis tool.

The presence of population structure was examined with the Structure software version 2.2. assuming the "no admixture" model. A list with the selected markers is available on request.

4 Results

The prevalence of the G1422A variant was not significantly different between cases and controls (OR=1.056; p=0.626). In obese men (but not women), homozygosity for the variant A allele was significantly associated with highest waist-to-hip ratio (WHR) and increased waist circumference (p=0.009 and p=0.008, respectively; values adjusted for age and body-mass-index). A trend for an association with increased fat mass was also observed (p=0.033).

All of the markers used to estimate the degree of population stratification were consistent with Hardy-Weinberg equilibrium (p>0.01) according to the analysis in the HWE-module of the MPAS software. This was true for the obese group as well as for the lean controls. (Fig. 2).

Although there were small differences in genotype frequencies when subjects were stratified by gender or by affection status (*i.e.* lean vs. obese) (Fig. 3), none of these reached significance (p>0.05; not shown).

Finally, the analysis with the Structure software package indicated that all individuals in the population stratification sample were in all likelihood derived from the same population and thus no population structure could be inferred (data not shown).

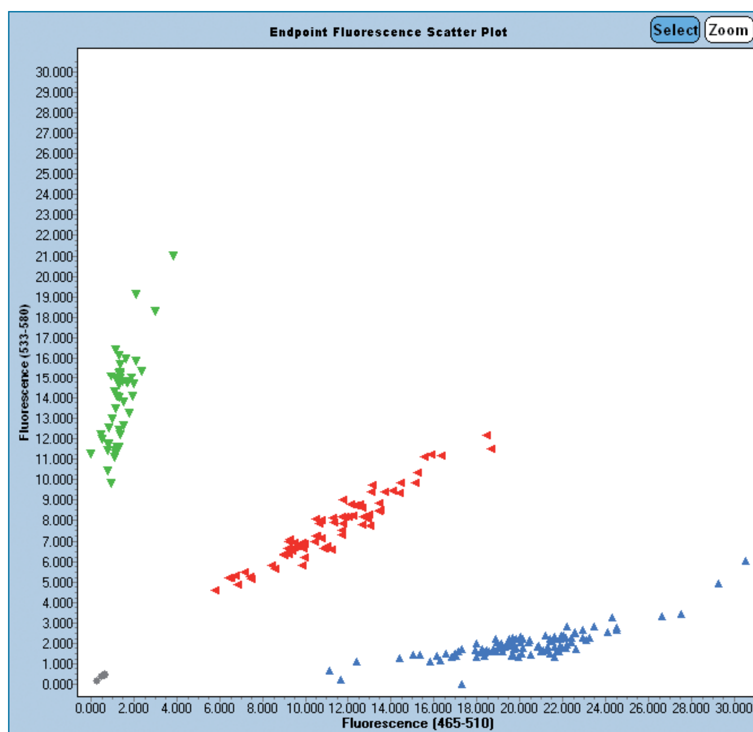

Figure 1: Typical results for a hydrolysis-probe assay for CNR1 gene variant G1422A (rs1049353), using the LightCycler® 480 Instrument. A fragment of the human CNR1 gene was amplified using the LightCycler® 480 Probes Master and subjected to endpoint analysis. Scatter plots consistently revealed wild-type (GG), mutant (AA) and heterozygote (GA) variants.

Hardy-Weinberg

Filter: All Samples

Filter

	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9	SNP10
observed Count Genotype AA	83	48	5	61	164	10	44	150	148	30
observed Count Genotype AB	173	139	86	158	142	106	144	137	137	109
observed Count Genotype BB	67	135	233	104	16	207	136	36	38	185
observed Frequency Allele A	52,5%	36,5%	14,8%	43,3%	73,0%	19,5%	35,8%	67,6%	67,0%	26,1%
observed Frequency Allele B	47,5%	63,5%	85,2%	56,7%	27,0%	80,5%	64,2%	32,4%	33,0%	73,9%
expected Count Genotype AA	88,9	42,9	7,1	60,7	171,5	12,3	41,5	147,8	145,1	22,0
expected Count Genotype AB	161,1	149,2	81,8	158,6	127,0	101,4	148,9	141,4	142,8	124,9
expected Count Genotype BB	72,9	129,9	235,1	103,7	23,5	209,3	133,5	33,8	35,1	177,0
Chi Inv	1,7612	1,5179	0,8637	0,0052	4,5002	0,6575	0,3562	0,3103	0,5275	5,2647
Hardy Weinberg equilibrium met (Chi inv < 6.63, alpha = 0.01)	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES

Figure 2: Hardy-Weinberg equilibrium test performed using the LightCycler® 480 Multiple Plate Analysis Software.

5 Conclusions

Comparative analysis of anthropometric measurements in research samples of adult obese individuals stratified by gender revealed meaningful differences between obese men with distinct genotypes of the CNR1 G1422A variant. Under a recessive model, we found three significant associations in the same direction viz. 1422A/A homozygotes are more abdominally obese, which adds evidence that these are true associations. Our data indicate that the absence of a CNR1 gene with the G-allele at position 1422 increases the risk for obesity in males. The fact that the associations were only seen in obese men can potentially be explained by gender differences in eating in general and fat ingestion in particular, as was previously suggested (7). It is unlikely that the observed associations are the result of population stratification as no evidence of population structure admixture was found. In conclusion, our data indicate that the G1422A polymorphism in the cannabinoid receptor-1 gene is associated with increased abdominal adiposity in obese men.

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About the author

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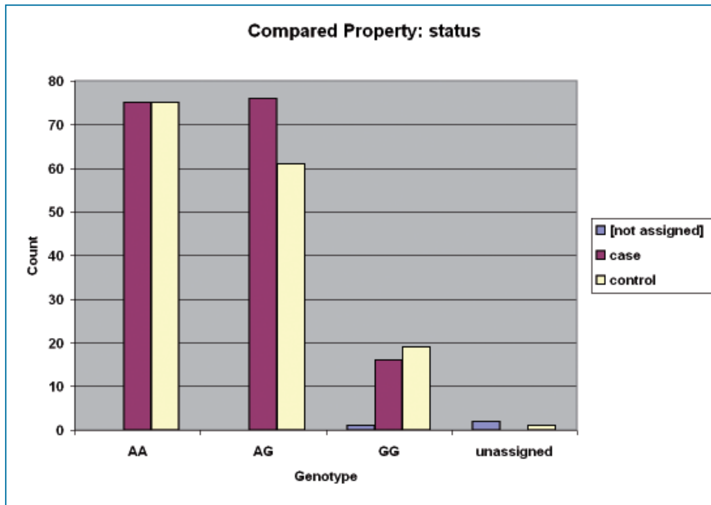

Figure 3: Parameter Comparison cases vs. controls --> bar graph

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Phenotype	Genotype	Mean ± SEM *	n	P (adjusted for age and body-mass-index)
Waist (cm)	G/G+G/A	123.3 ± 0.6	422	
	A/A	127.3 ± 2.2	33	0.008
Waist-to-Hip ratio	G/G+G/A	1.09 ± 0.006	420	
	A/A	1.15 ± 0.022	32	0.009
Fat mass (kg)	G/G+G/A	52.2 ± 0.8	432	
	A/A	57.5 ± 3.2	36	0.033
Fat mass %	G/G+G/A	42.1 ± 0.3	432	
	A/A	44.7 ± 1.0	36	0.218

Table 1: Associations under a recessive model between G1422A genotypes and anthropometric values in obese men.

*** standard error of the mean**

Melting Curve Genotyping: Correlation Between SNP Alleles and Smoking Behavior

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1 Introduction

Smoking behavior is influenced by both genetic and environmental factors. Moreover, smoking initiation and persistence have a heritability of at least 50%. A large body of work has been dedicated to associating multiple genetic markers of neurobiological pathways previously linked to addiction and smoking (such as the central dopaminergic, serotoninergic, and nicotinergic pathways) with different aspects of smoking behavior.

In order to investigate the feasability of future genetic analyses, we genotyped 14 SNPs in 272 research samples of addicted smokers using HybProbe assays on the LightCycler® 480 Real-Time PCR System. Statistical analysis was done using the LightCycler® 480 Multiple Plate Analysis Software.

2 DNA Preparation and Sample Setup

Research blood samples were taken from volunteers who had given informed consent for analysis of target genes presumably involved in nicotine addiction. For this study DNA was prepared using either a manual high-salt method (236 samples) or the MagNA Pure Compact Instrument with the MagNA Pure Compact Nucleic Acid Isolation Kit I (36 samples).

3 Assay Design and PCR Protocol

PCR primers and HybProbe probes for the analysis of 14 different SNPs were designed by Tib Molbiol (Berlin). As a general rule and starting point, we used 10 pmol of each primer and 3 pmol of each HybProbe probe (anchor and sensor). PCR volumes were 10 µl, and we amplified 55 cycles with a touchdown PCR profile (lowering the annealing temperature from 65°C in the first cycle to 55°C in the 10th cycle in 1°C/cycle steps.

4 Assay Optimization and Evaluation

Overall, we achieved robust genotyping for 12 of 14 genotyping assays with our standard approach (Figure 1), relying mainly on a touchdown PCR protocol and asymmetric amplification (preferentially amplifying the template DNA strand complementary to the anchor and sensor probes). For two assays (COMT and MAOA), we had to modify the conditions in order to get satisfactory results. In the case of COMT, the standard approach gave melting peaks of very different heights for the mismatch (low temperature melting) and the perfect match (high temperature melting) alleles (Figure 2). Changes in primer or HybProbe probe concentrations did not change this pattern. At this stage, we were able to distinguish only between homozygous BB genotypes (in purple) in all other genotypes (AA and AB; yellow and blue samples) and thus had not obtained enough discrimination power. We therefore decided to move the unlabeled forward primer 50 bases upstream and, by this simple measure, greatly improved the robustness of the genotyping assay. Another assay round gave us reliable genotypes for 268 samples (1.5% technical failures).

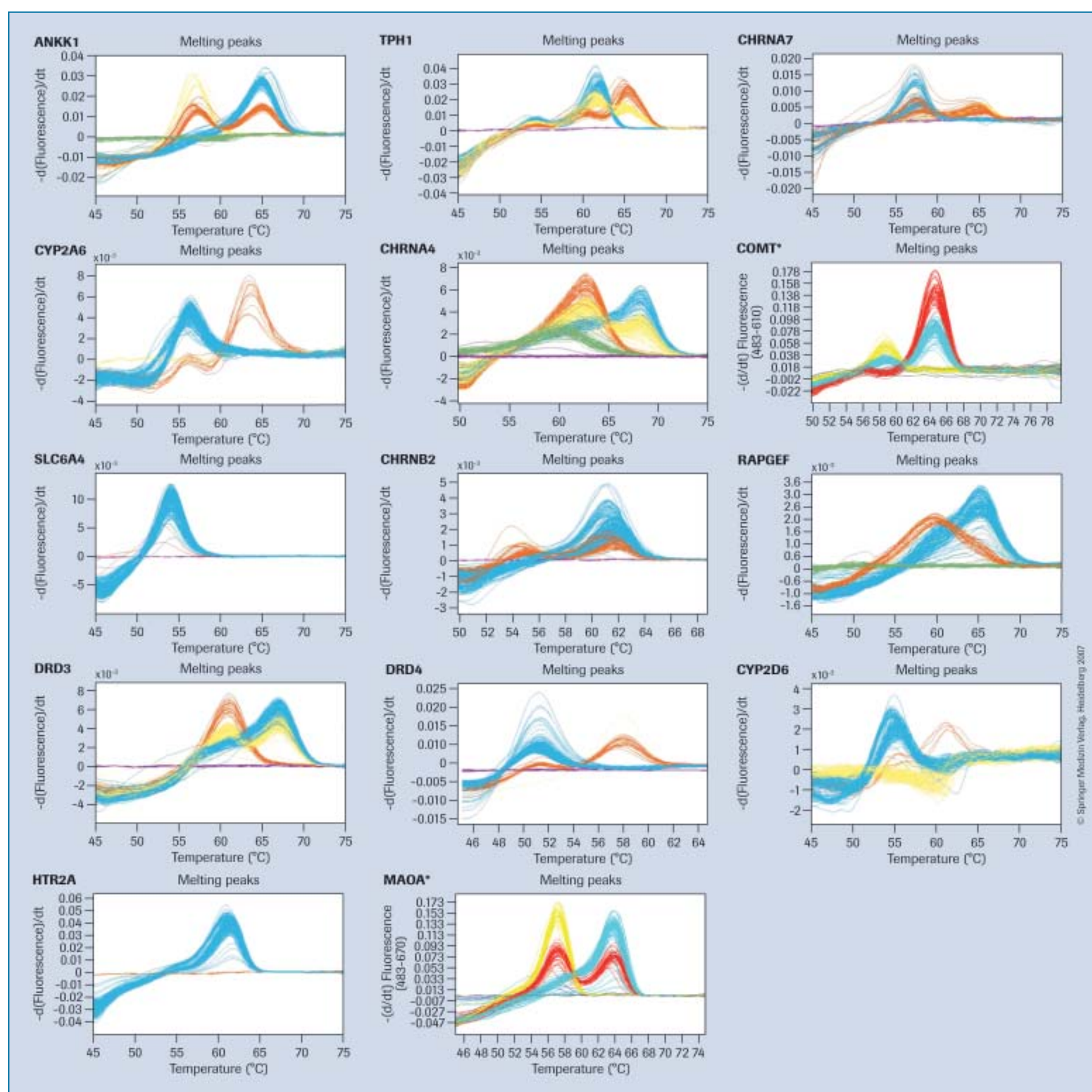

Figure 1: Overview of 14 SNP genotyping assays performed with HybProbe probes.
For targets marked with as asterisk (COMT and MAOA), assay conditions had to be optimized as shown in Fig. 2.

In case of the MAOA polymorphism, again, melting curves for the high temperature melting peak were polymorphic and did not allow accurate grouping of genotypes (Figure 2b). A skewed melting profile did not reliably distinguish between AB genotypes (yellow traces) and BB genotypes (red traces). In this case, we observed much better melting profiles in 20 µl volume assays, and therefore carried out a technically identical assay in a doubled PCR volume. Using this approach, we were able to reliably genotype all samples for MAOA (except for 1.9% technical failure probably due to poor DNA quality).

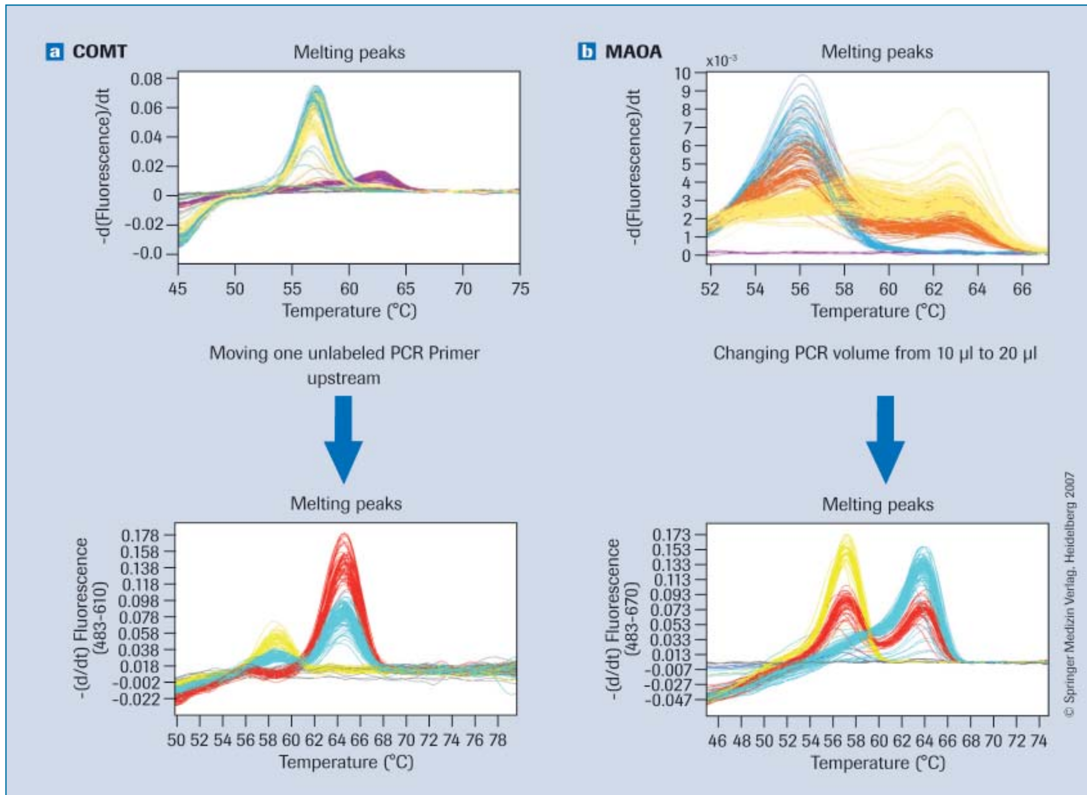

Figure 2: Optimization strategies for HybProbe assays targeting the COMT and MAOA genes.

5 Multiple Plate Genotyping and Statistic Analysis

To extract biologically meaningful information from genotyping studies, a high number of samples need to be tested and data derived from them compiled. In case of the LightCycler® 480 System, the LightCycler® 480 Multiple Plate Analysis Software is available for this purpose, as providing a powerful toolbox to easily compile multiple-run data and extract statistic paramenters. For genetic variation research, it comprises functionalities for haplotype and genotype counts, enabling quick access to frequencies and distributions for individual or combined SNPs based on calls obtained with the LightCycler® 480 Instrument Software. We tested the utility of this tool by analyzing allele combinations of 4 adjacent tagSNPs in the DRD3 gene.

After genotyping experiments had been performed on the LightCycler® 480 Instrument and analyzed using the Genotyping module of LightCycler® 480 Software, Version 1.5, the individual experiments containing the analyzed results were exported from the LightCycler® 480 database as ixo-files (individually or in a single batch) and then imported in the LightCycler® 480 Multiple Plate Analysis Software. Files and samples were edited appropriately (names, type etc.).

The preference tab in the provided import interface allowed assignment of known properties (in our study *e.g.*, smoking intensity) to each sample. The software then deduced study data like *allele frequencies*, and *genotype counts*, providing them as a compiled spreadsheet for the whole data set. The latter finally summarized phenotypic, genotypic and haplotypic data for each sample (see Fig. 3 for a summary of the workflow). To check for data contingency, an automated check for fulfilment of Hardy-Weinberg equilibrium conditions was performed. This allowed us to conclude that the chosen sample cohorts had no selection bias and that the genotyping had been done correctly (see Fig. 4).

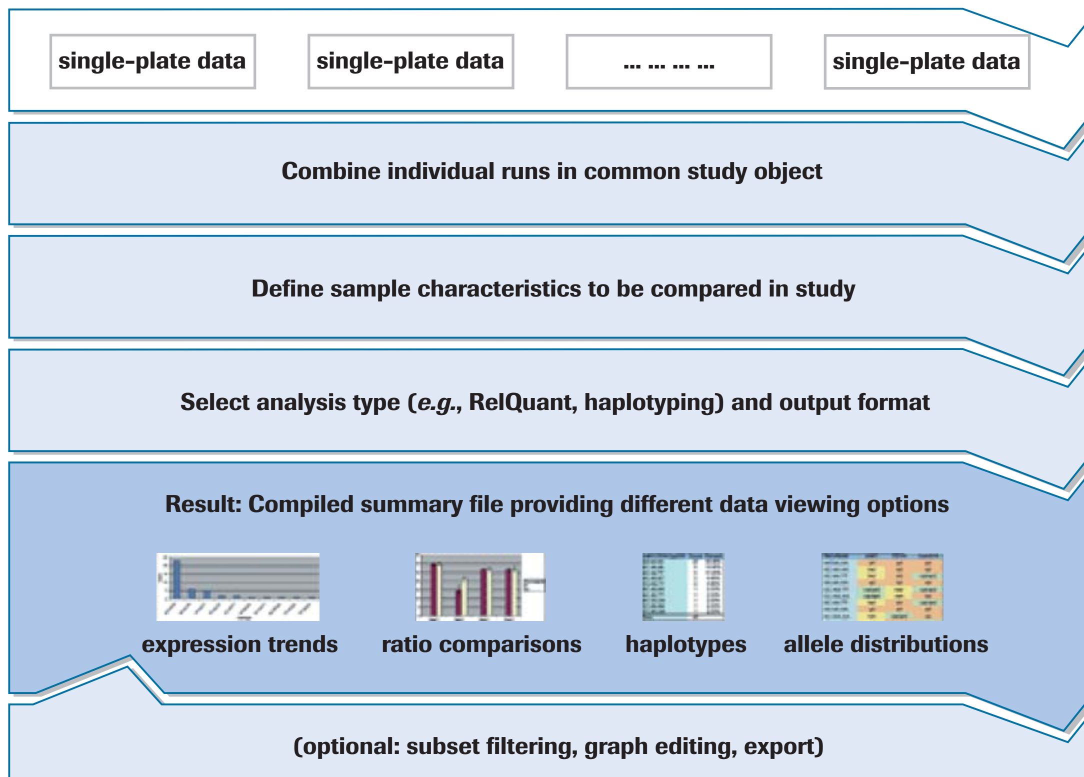

Figure 3: Overview of the LightCycler® 480 Multiple Plate Analysis Software workflow.

Supported by these relevant quality measures, the Multiple Plate Analysis Software provided conclusive and accurate genotyping and haplotyping data for our study. Exemplified for DRD3, we could directly conclude which haplotypes are correlated with smoking intensity (Figure 5). Usually, structured analysis of our data set would have taken several hours. Supported by the Multiple Plate Analysis Software tool, analysis and basic visualization was accomplished in less than one hour.

6 Summary and Conclusion

Since Real-Time PCR is very often used to validate results obtained from large-scale studies (*e.g.*, data obtained on microarrays), the scope and size of such studies has significantly increased over the recent years. Using the HybProbe probe assays on the LightCycler® 480 System in combination with LightCycler® 480 Multiple Plate Analysis Software, we could establish a fast and straightforward workflow and greatly improve and accelerate the generation of large data sets obtained from multiple plates and instrument runs. Large amounts of high quality data could be generated within short turnaround time. In summary, the LightCycler® 480 System in combination with HybProbe probe melting curve assays provides a straightforward workflow to design, set up, carry out and analyse extended genotyping studies. Compiled analysis of extended Real-Time PCR studies has often been hampered by the need to carry out multiple, error-prone and time-consuming export-import or copy-paste operations into third-party applications. In contrast to this, quality control – not only on the level of a single experiment but also for extensive studies on the meta-data level – is implemented in the LightCycler® 480 Multiple Plate Analysis Software in order to certify high quality results over time. This software therefore now provides researchers with an efficient and easy-to-use toolbox, circumventing the risk of data loss or miss-assignment and giving easy access to meaningful study results.

	S9G B	S9G RS	SNP16	SNP16	SNP17	SNP17	SNP14	SNP14
observed Count Genotype AA	111	127	65	66	27	29	146	169
observed Count Genotype AB	89	92	102	112	86	111	67	67
observed Count Genotype BB	17	19	51	55	105	101	5	9
observed Frequency Allele A	71,7%	72,7%	53,2%	52,4%	32,1%	35,1%	82,3%	82,7%
observed Frequency Allele B	28,3%	27,3%	46,8%	47,6%	67,9%	64,9%	17,7%	17,3%
expected Count Genotype AA	111,4	125,8	61,7	63,9	22,5	29,6	147,8	167,4
expected Count Genotype AB	88,1	94,5	108,6	116,2	95,0	109,7	63,4	70,3
expected Count Genotype BB	17,4	17,8	47,7	52,9	100,5	101,6	6,8	7,4
Chi inv	0,0206	0,1660	0,7938	0,3101	1,9747	0,0315	0,7023	0,5259
Hardy Weinberg equilibrium met	YES	YES	YES	YES	YES	YES	YES	YES

Figure 4: Four SNPs were genotyped in two independent sample cohorts. The software´s Hardy-Weinberg analysis tab shows all relevant data for observed genotypes, expected genotypes and allele frequencies in all groups. Moreover, a chi-square test is performed and interpreted for every group, allowing an unambiguous interpretation of data obtained for dichotomous SNPs.

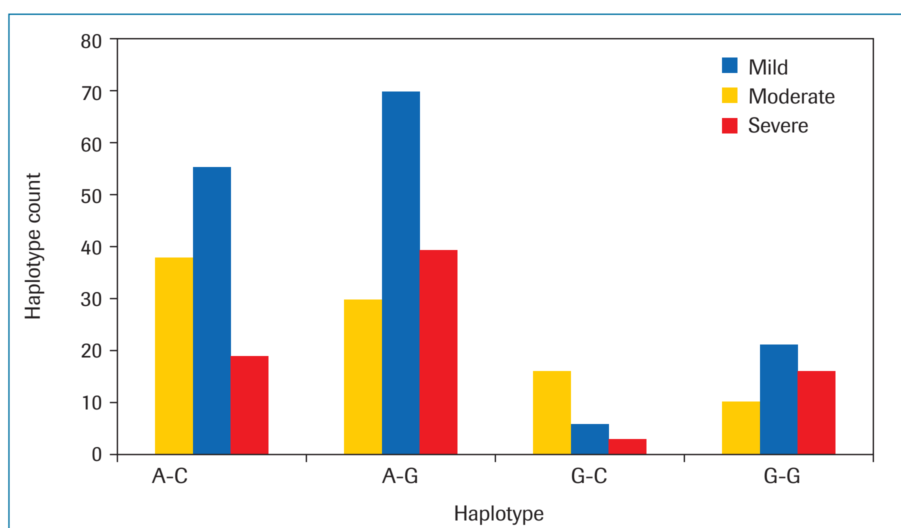

Figure 5: Two neighboured SNPs in the DRD3 gene were genotyped and results transferred to the LightCycler® 480 Multiple Plate Analysis Software to deduce haplotypes. Evidently, the haplotype A-C is overrepresented in moderate and severe smokers, while the haplotype G-C is rare in these subgroups.

7 References

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Methylation-Sensitive High Resolution Melting (MS-HRM) on the LightCycler® 480 System

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Abstract

High Resolution Melting Curve Analysis allows distinction of amplicons based on their sequence dependent melting characteristics. Sodium bisulfite deaminates unmethylated cytosines into uracil while 5-methylcytosine is resistant to that modification. Therefore PCR products derived from bisulfite modified DNA template have methylation-dependent base compositions, with thymines incorporated at the unmethylated cytosines sites and cytosines at the 5-methcytosine residues. High Resolution Melting technology enables differentiation of those PCR products and hence investigation of the methylation status of regions of interest. In this article, we provide a protocol for Methylation-Sensitive High Resolution Melting (MS-HRM) on the LightCycler® 480 System allowing for highly sensitive, labor and cost-efficient single locus methylation studies.

1 Introduction

Epigenetics in disease studies

The mechanisms of gene expression regulation that do not involve changes of the DNA primary sequence are referred to as epigenetic mechanisms of regulation of transcription. Covalent histone modifications and the enzymatic addition of methyl group to the 5th carbon of a cytosine in DNA helix are the most studied epigenetic mechanisms that regulate gene expression.

Methylation of cytosines in human DNA occurs almost exclusively at the palindromic cytosine followed by guanine (CpG sites). Regions of DNA showing relatively high numbers of CpG sites are called CpG islands (CGI). Most of the CGIs in the human genome are methylated, with the exception of CGIs located in the 5' regulatory region of protein coding genes. Approximately 60% of protein coding genes contain CGIs, and *de novo* methylation of CGIs in promoters reduces transcriptional activity[1].

Methylation depedent regulation of gene expression has wide physiological implications and plays a role in tissue differentiation, silencing of transposable elements in the genome, allele specific expression of the genes (genomic imprinting), X chromosome inactivation and environmental dependent gene expression regulation.

Changes of the normal methylation makeup of the cell have long been recognized to occur in the pathology of many diseases and have been most widely studied in neoplastic disorders. The transcriptional silencing of the tumor suppressor genes by methylation of promoter CGI has been proposed as the additional "hit" in Knudson's two hit theory of tumor suppressor deactivation[2, 3].

Methylation changes have been shown to be tumor specific, arising in early stages of carcinogenesis and accumulating as tumors progress. The specificity of methylation changes allows to predict a tumor´s phenotype on the bases of its methylation signature. The methylation signature of the tumor may therefore be valuable in clinical practice for both prediction of response to a treatment and establishment of the prognosis. Furthermore, if tumor DNA can be detected in an individual´s blood flow or other body fluids, detection of cancer specific methlyation markers could possibly be used in early and non-invasive diagnosis of neoplastic disease[4].

High Resolution Melting

High Resolution Melting analyses have initially been developed for DNA sequence alteration screening and have been proven to be a powerful methodology in both detection of new sequence alternations and in genotyping studies. Furthermore the sensitivity of HRM analyses has been shown to be superior to conventional sequencing in many cases[5].

Different amplicons have a sequence dependent melting temperature. Melting temperature is defined as a temperature at which half of the PCR product denatures from double stranded into single stranded coils. HRM protocols make use of a new class of saturating, DNA-binding dyes, which allow to monitor PCR product melting and therefore investigation of melting temperatures and melting curve shapes. DNA intercalating dye at low temperatures binds to double stranded DNA emitting high level of florescence which can be detected by HRM fluorescence acquisition instruments. When the temperature is gradually increased the high levels of fluorescence remain stabile until the point when the double stranded PCR product melts in to single stands. At that point a sharp drop in fluorescence is observed as a consequence of the inability of DNA intercalating dye to bind (and emit fluorescence) to single stranded DNA. The drop in fluorescence indicates the specific melting temperature of the amplicon and the graphic illustration of changes in fluorescense over the temperature gradient is referred to as the amplicon´s melting profile.

Methylation-Sensitive High Resolution Melting (MS-HRM)

The introduction of sodium bisulfite DNA treatment enabled the use of PCR amplification in methylation studies. Sodium bisulfite deaminates cytosines, whereas 5-methylcytosines are resistant to this modification. Therefore the PCR product amplified from bisulfite modified template has a methylation-sensitive sequence with thymines at the cytosines sites and cytosines at the 5-methylcytosines sited. The PCR product derived from unmethylated template (low GC content) will require less energy to melt (have a lower melting temperature) than the PCR product originating from methylated version of the same template (relatively higher GC content). When subjected to a temperature gradient in the presence of saturating dyes, methylated and unmethylated amplicons will display different melting profiles. The methylation status of an unknown sample can be therefore investigated by comparing its melting profile with the melting profiles of the PCR products derived from the methylated and unmethylated variants of the same template [6].

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2 Materials and Methods

Sample and Nucleic Acid Isolation

Genomic DNA for HRM analysis should be extracted using a good practise method (*e.g.*, High Pure PCR Template Preparation Kit).

Bisulfite Modification

Bisulfite modification can be performed as described elsewhere[7], or using commercially available bisulfite modification kits.

Primer design

In MS-HRM protocols, the methylation status of the sample is assessed in a post-PCR, close-tube manner.Therefore the protocol utilizes primers that amplify both the methylated and unmethylated template. To score the methylation status of the screened sample both methylated and unmethylated templates have to be amplified in the same proportion as it was present in the original sample. The guidelines for primer design used in amplification of bisulfite modified DNA for post PCR methylation screening protocols were published elsewhere[8] and advise to avoid any CpG dinuclotides in the primers sequence and, if this can not be achieved, to mismatch C positions in CpG sites with T. Others and we have shown that primers designed according to this strategy may lead to preferential amplification of unmethylated (GC-poor) template and as a consequence to underestimation of methylation levels in the screened material[9, 10].

We have addressed the PCR bias issue and developed a new strategy for primer design in methylation studies[11]. Our strategy assumes inclusion of a limited number of CpG sites in the primer sequences. The specificity of binding of primers with a limited number of CpGs to methylated and unmethylated templates is PCR annealing temperature depended. At low temperatures primers bind to both templates with comparable efficiency and are not able to correct for PCR bias occurring at the strand polymerization step. However at higher melting temperature the primers will preferentially bind to the methylated template enabling robust amplification and elimination of PCR bias[6, 11, 12]. The new strategy for primer design allows for detection of the methylated template with 0,1-1% sensitivity, which is the sensitivity of methylation-specific PCR.

Based on these new findings, updated guidelines for primer design have been established and are outlined below (an example is given in Fig.1):

1. Primers should usually contain one or two CpGs dinuclotides each.
2. The CpGs should be placed as close as possible to the 5’ end of the primer.
3. The melting temperature of the primers should not differ by more than one degree.
4. The 3’ end of the primer should contain one or more Ts specific to non-CpG Cs, to ensure amplification of only bisulfite converted template.
5. The primers should meet standard parameters for primer design, *e.g.,* no secondary structure or primer dimer formation.
6. The preferred length of the amplified sequence should be around 100bp to reduce the complexity of the melting profile.

Methylated and unmethylated controls

In MS-HRM experiments, to correctly score the methylation status of screened samples the melting profile of the PCR product has to be compared to the melting profile of amplicons derived from both a methylated and an unmethylated reference. The choice of the proper references is critical for the MS-HRM protocol and has to be performed prior to experiments where unknown samples are to be tested. The methylation status of different loci can vary in different tissues. Therefore before choosing a DNA source for methylated and unmethylated references the methylation status of the control templates has to be unambiguously confirmed. Treatment of genomic DNA with *M.SssI* enzyme can be performed to obtain methylated reference but repeated rounds of treatment may be necessary to obtain fully methylated DNA. As unmethylated reference, some laboratories use whole genome amplified DNA. The use of commercially available methylated and unmethylated controls is convenient, but even in those, the assessment of methylation status of the locus of interest has to be performed.

PCR amplification

Standard PCR protocols can be used for amplification of template DNA in MS-HRM applications.The required saturating HRM dye (*e.g.,* LightCycler® 480 ResoLight), which allows highly sensitive melting monitoring, can be used as an additive to the PCR mix. For more convenience, Roche Applied Science offers a LightCycler® 480 High Resolution Melting Master, including the saturating DNA dye and all PCR reagents. However the amplification of bisulfite modified DNA is not as robust as amplification of gDNA and can be enhanced by elevating the Mg^{+2} concentration. In our practice the Mg^{+2} concentration in the range of 2.5 – 3.5 mM is optimal in most of the cases.

The PCR cycling parameters should be optimized individually for each amplification to ensure optimal PCR efficiency (see Fig.1 for example).

Identification of PCR bias and optimization of annealing temperature for PCR

The extent of PCR bias can be identified as described elsewhere[9]. Briefly, the PCR amplification should be performed using 1:1 mixes of methylated and unmethylated template, and the HRM results analysis of such a sample should display equal amount of PCR products for methylated and unmethylated templates (see *Analysis of results* for details).

The mixes of methylated and unmethylated template in the range suitable for the experiments should also be used for optimization of the annealing temperature of PCR amplification, allowing to access the sensitivity of the assay and to investigate the annealing temperature at which the PCR bias is compensated.

High Resolution Melting equipment

The Roche LightCycler® 480 System is an ideal platform for MS-HRM experiments since it allows for PCR amplification of the samples in combination with subsequent HRM analyses. Monitoring of amplification in real-time facilitates exclusion of samples where the amplification failed, providing an additional QC step in HRM projects.

Parameters of HRM analysis

The temperature gradient for HRM analysis should be wide enough to include the melting temperatures of both methylated and unmethylated PCR products derived from bisulfite modified template. Its range needs to be empirically determined.

The LightCycler® 480 Instrument calculates automatically the time needed for a given number of fluorescence acquisition points per °C and designs the temperature gradient. In most cases, we found 50 fluorescence acquisitions per °C to be sufficient for obtaining high quality melting data.

3 Analysis of Results

Derivative peaks (T_m calling)

High resolution melting profiles obtained from HRM analysis can be transformed into melting peaks. The transformation is calculated by plotting the divided first negative derivative of fluorescence (-dF) and minus first derivative of temperature (-dT) over the temperature gradient (Figure 1). After this transformation the top of the peak represents the sharpest drop of fluorescence in the temperature gradient and indicates the melting temperature of the amplicon. This calculation allows for fast forward comparison of the melting profiles of the test samples with the melting profiles of the methylated and unmethylated references.

Gene scanning (direct visualization of melting curves)

HRM curves cannot be directly compared after HRM scan, because the system is able to detect even small differences in the amounts of the PCR product subjected to HRM, making even replicates of the same sample visually different. Therefore, newly developed algorithms for HRM data analysis, enabling direct comparison of HRM curves, have to be applied. These algorithms allow normalization of the fluorescence data, therefore enabling comparison of the melting profiles solely based on the shapes of the curves. Furthermore, the melting profiles of test samples can be compared to standards with known methylated to unmethylated template ratio to investigate their methylation level (Figure 2)[6].

Heterogeneously methylated samples

Some loci in the genome display heterogeneous methylation patterns. At heterogeneously methylated loci different CpG dinuclotides display different methylation status along the locus sequence. HRM technology allows distinction of samples with heterogeneously methylated loci of interest from samples with fully methyaletd/unmethylated loci. The PCR products derived from heterogeneously methylated loci display many minor differences in the base sequence and as a consequence can cross hybridize and form heteroduplexes. The heteroduplexes of PCR products have different melting temperatures. Therefore PCR product derived from heterogeneously methylated loci will not display consistent melting temperature but will melt along the temperature gradient. Those samples can be distinguished by HRM scanning and can be investigated further by using DNA sequencing technology.

Figures descriptions:

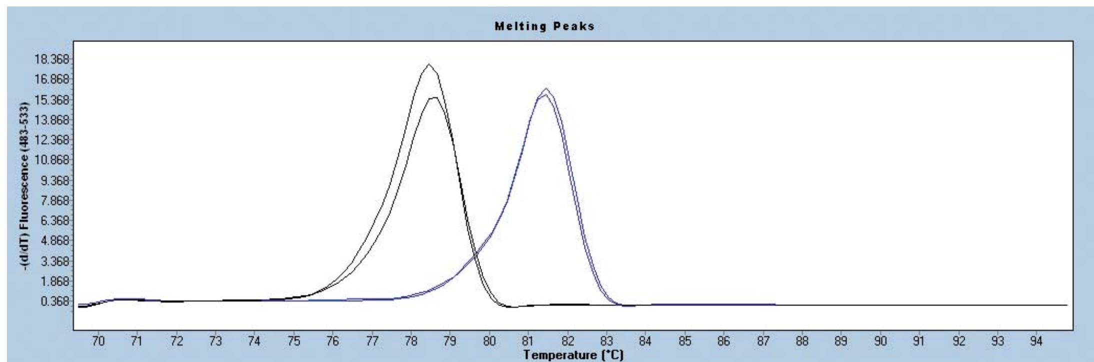

Figure 1: A portion of the 5' regulatory sequence of the *APC* gene (chr5:112,101,343-112,101,436 UCSC Genome Browser v191) was amplified with primers
F: 5'-G**CG**GAGAGAGAAGTAGTTGTG TAATT-3', and R: 5'-TA**CG**CCCACACCCAACCAATC-3'. The PCR mix contained 250 nM of each primer, 3 mM of Mg^{+2} and 1× LightCycler® 480 High Resolution Melting Master. The PCR cycling conditions were: initial incubation 95°C 10 min, and 50 cycles of: 95°C 5 s, 64°C 5 s, 72°C 10 s. Products were prepared for HRM analysis by heating to 95°C for 1 min, rapid cooling down to 70°C and incubation for 1 min. HRM analyses parameters were: temperature gradient from 70 - 95°C with 50 fluorescence acquisition points per °C. The HRM scans were analyses using the LightCycler® 480 Software´s Tm calling module. Two distinct peaks were obtained for PCR products originating from methylated (blue) and unmethylated (black) template, respectively.

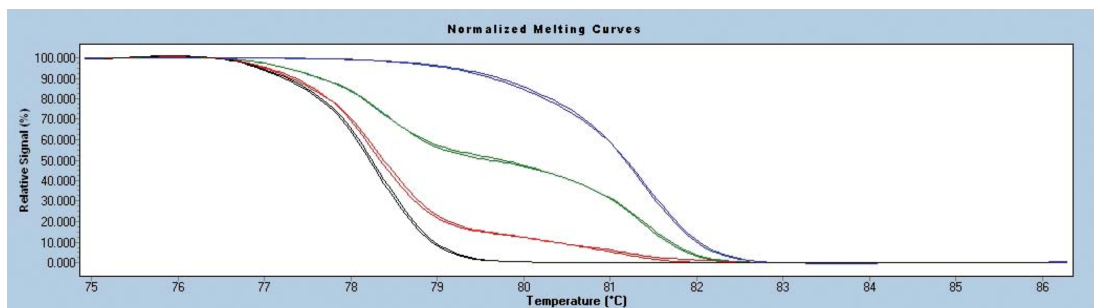

Figure 2: The *APC* 5' regulatory sequence was amplified from mixes of methylated and unmethylated bisulfite modified template (for details see Figure 1). The PCR products derived from the mixes where subsequently analyzed using LightCycler® 480 Gene Scanning Software. Distinct melting curves were obtained for each mix, displaying two melting transitions for samples containing both methylated and unmethylated template: 10% (green) and 1% (red) of methylated DNA in unmethylated background and 100% methylated (blue) and unmethylated (black). References with know ratio of methylated to unmethylated template served as standards for estimating the methylation level of unknown samples on the basis of the similarities of melting profiles.

4 Conclusion

With an increasing role of epigenetics and in particular DNA methylation in human disease, both research and diagnostic settings are in need for reliable and efficient methylation detection methodologies. Current methods for single locus methylation screening are based upon different principles for assay design and have many pitfalls, which make performance of the methylation screening experiments very difficult.

The MS-HRM (Methylation-Sensitive High Resolution Melting) protocol described in this article for use on the LightCycler® 480 System allows for detection of the methylation status of loci of interest following bisulfate modification of template DNA in less than 3 hours. In the protocol, both PCR amplification and HRM analysis of the PCR product are performed in the same tube, which eliminates the risk of contamination of laboratory settings with PCR products. The analysis of MS-HRM results does not require complicated calculations. The protocol can be optimized to give both qualitative and semi-quantitative results. Moreover the methodology enables identification of heterogeneously methylated loci, within which methylation status of single CpG sites can be determined by subsequent DNA sequencing. The results obtained via the MS-HM protocol are highly reproducible. The method has high specificity rates due to the fact that the MS-HRM protocol in a single reaction not only detects methylation of a given locus but at the same time confirms the unmethylated status of methylation-devoid loci.

Recently developed by us, a novel primer design strategy for use in combination with High Resolution Melting technology now allows controlling of the PCR bias, which otherwise might lead to significant underestimation of the methylation level of the screened material. The sensitivity of the new primer design system in the detection of methylation is similar to the sensitivity provided by other technologies in the field.

To summarize, MS-HRM is a fast, cost and labor efficient research method for single locus methylation studies on the LightCycler® 480 System.

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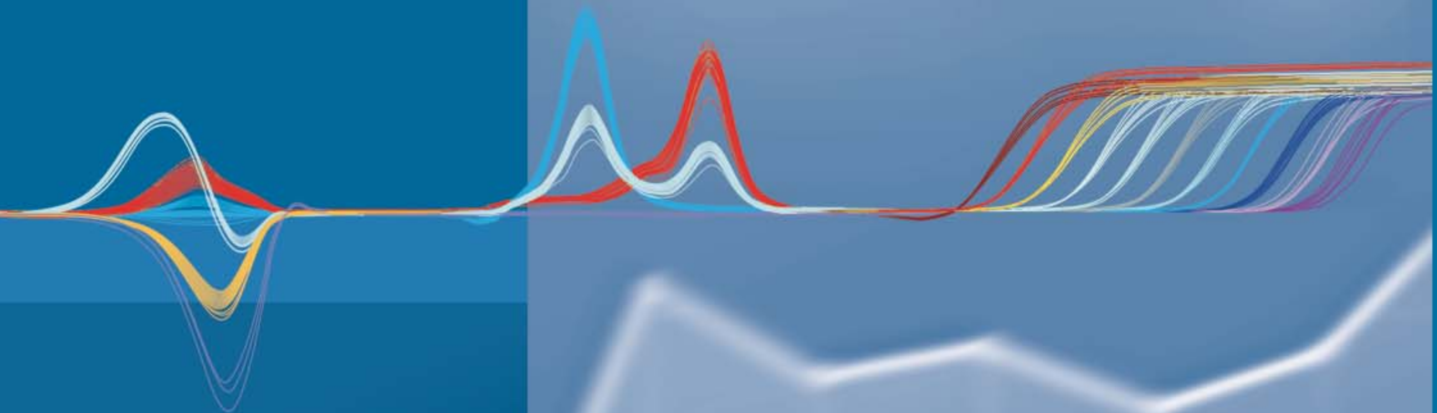

Ordering Information

LightCycler® 480 Instruments and Additional Products

Product	Cat. No.	Pack Size
LightCycler®480 Instrument II, 96-well	05 015 278 001	1 Instrument[1]
LightCycler®480 Instrument II, 384-well	05 015 243 001	1 Instrument[1]
LightCycler® 480 Block Kit 96 Silver	05 015 219 001	1 Kit[2]
LightCycler® 480 Block Kit 384 Silver	05 015 197 001	1 Kit[2]
LightCycler® 480 Bar-Code Scanner	04 710 606 001	1 Scanner
LightCycler® 480 Xenon Lamp	04 686 136 001	1 Lamp
LightCycler® 480 Software, Version 1.5	04 994 884 001	1 Software Package
LightCycler® 480 LIMS Interface Module	05 066 310 001	1 Software Package
LightCycler® 480 Gene Scanning Software	05 103 908 001	1 Software Package
LightCycler® 480 Multiple Plate Analysis Software	05 075 122 001	1 Software Package
LightCycler® 480 Multiwell Plate 96, white	04 729 692 001	50 Plates / 50 Foils
LightCycler® 480 Multiwell Plate 384, white	04 729 749 001	50 Plates / 50 Foils
LightCycler® 480 Multiwell Plate 96, clear	05 102 413 001	50 Plates / 50 Foils
LightCycler® 480 Multiwell Plate 384, clear	05 102 430 001	50 Plates / 50 Foils
LightCycler® 480 Sealing Foil	04 729 757 001	50 Foils

[1] Instrument package includes LightCycler® 480 Instrument, LightCycler® 480 thermal block cycler unit (96- or 384-well), LightCycler® 480 Software 1.5, LightCycler® 480 Instrument Operator's Manual, LightCycler® 480 Xenon Lamp (spare lamp). A Pentium desktop PC is supplied with the instrument.

[2] Kit package includes LightCycler® 480 thermal block cycler unit (96- or 384-well), block cycler cover, storage box.

LightCycler® 480 Reagents and RT-PCR Products

Product	Cat. No.	Pack Size
LightCycler® 480 SYBR Green I Master (2× concentrated)	04 707 516 001 04 887 352 001	5 × 1 ml (500 × 20 µl reactions) 10 × 5 ml (5000 × 20 µl reactions)
LightCycler® 480 Probes Master (2x concentrated)	04 707 494 001 04 887 301 001 04 902 343 001	5 x 1 ml (500 × 20 µl reactions) 10 × 5 ml (5000 x 20 µl reactions) 1 × 50 ml (5000 × 20 µl reactions)
LightCycler® 480 Genotyping Master (5× concentrated)	04 707 524 001	4 × 384 µl (384 × 20 µl reactions)
LightCycler® 480 High Resolution Melting Master	04 909 631 001	5 × 1 ml (500 × 20 µl reactions)
LightCycler® 480 RNA Master Hydrolysis Probes	04 991 885 001	500 × 20 µl reactions
LightCycler® 480 CYAN 500 Labeling Reagent	04 764 153 001	1 vial
LightCycler® 480 Control Kit	04 710 924 001	1 Kit (3 control runs)
LightCycler® RNA Pre-Amplification Kit	05 190 894 001	32 reactions
Transcriptor First Strand cDNA Synthesis Kit[1]	04 379 012 001 04 896 866 001 04 897 030 001	1 Kit (50 reactions) 1 Kit (100 reactions) 1 Kit (200 reactions)
Universal ProbeLibrary Set, Human Reference Gene Assays	05 046 114 001	1 set
Universal ProbeLibrary Set, Human	04 683 633 001	1 set[2]
Universal ProbeLibrary Set, Mouse	04 683 641 001	1 set[2]
Universal ProbeLibrary Set, Rat	04 683 650 001	1 set[2]
Universal ProbeLibrary Extension Set	04 869 877 001	1 set[2]
Universal ProbeLibrary, Human PBGD Gene Assay	05 046 149 001	500 reactions
Universal ProbeLibrary, Human HPRT Gene Assay	05 046 157 001	500 reactions
Universal ProbeLibrary, Human ACTB Gene Assay	05 046 165 001	500 reactions
Universal ProbeLibrary, Human PGK1 Gene Assay	05 046 173 001	500 reactions
Universal ProbeLibrary Human GUSB Gene Assay	05 190 525 001	1 set[2]
Universal ProbeLibrary Human TBP Gene Assay	05 189 284 001	1 set[2]
Universal ProbeLibrary Human G6PD Gene Assay	05 046 246 001	1 set[2]
RealTime *ready* Human Reference Gene Panel, 96	05 339 545 001	2 plates

[1] For detailed information, **visit www.roche-applied-science.com/pcr**

[2] For detailed information, visit **www.universalprobelibrary.com**

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This LightCycler® 480 Real-Time PCR System is a real-time thermal cycler licensed for use in research, in vitro diagnostics and other applied fields under U.S. Patent No. 6,814,934 and corresponding claims in its non-U.S. counterparts, and under one or more of U.S. Patents Nos. 5,038,852, 5,656,493, 5,333,675, 5,475,610, 5,602,756, 6,703,236, 7,238,517 or corresponding claims in their non-U.S. counterparts, owned by Applera Corporation. No right is conveyed expressly, by implication, or by estoppel under any other patent claim, such as claims to apparatus, reagents, kits, or methods such as 5' nuclease methods.

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