# Real-Time PCR
## Applications Guide

BIO-RAD

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# Overview of Real-Time PCR

# 1. Overview of Real-Time PCR

Nucleic acid amplification and detection are among the most valuable techniques used in biological research today. Scientists in all areas of research — basic science, biotechnology, medicine, forensic science, diagnostics, and more — rely on these methods for a wide range of applications. For some applications, qualitative nucleic acid detection is sufficient. Other applications, however, demand a quantitative analysis. Choosing the best method for your application requires a broad knowledge of available technology.

This guide provides an introduction to many of the technical aspects of real-time PCR. It includes guidelines for designing the best real-time PCR assay for your experiments and explains how real-time PCR data are used in various applications. In Sections 5–7, we present sample protocols and data that demonstrate the use of real-time PCR in specific applications, namely, gene expression analysis, allelic discrimination, and genetically modified organism (GMO) detection.

We hope that this guide will give you the information you need to bring this powerful technique to your bench — and beyond.

## 1.1 Key Concepts of Real-Time PCR

### 1.1.1 What Is Real-Time PCR?

In conventional PCR, the amplified product, or amplicon, is detected by an end-point analysis, by running DNA on an agarose gel after the reaction has finished. In contrast, real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses, that is, in “real time”.

Real-time detection of PCR products is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently labeled sequence-specific primers or probes. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor the fluorescence as amplification occurs. The measured fluorescence reflects the amount of amplified product in each cycle.

The main advantage of real-time PCR over conventional PCR is that real-time PCR allows you to determine the starting template copy number with accuracy and high sensitivity over a wide dynamic range. Real-time PCR results can either be qualitative (presence or absence of a sequence) or quantitative (number of copies of DNA). Real-time PCR that is quantitative is also known as qPCR. In contrast, conventional PCR is at best semi-quantitative. Additionally, real-time PCR data can be evaluated without gel electrophoresis, resulting in reduced experiment time and increased throughput. Finally, because reactions are run and data are evaluated in a closed-tube system, opportunities for contamination are reduced and the need for postamplification manipulation is eliminated.

## 1.1.2 How Real-Time PCR Works

To understand how real-time PCR works, let's start by examining a sample amplification plot (Figure 1.1). In this plot, the PCR cycle number is shown on the x-axis, and the fluorescence from the amplification reaction, which is proportional to the amount of amplified product in the tube, is shown on the y-axis.

The amplification plot shows two phases, an exponential phase followed by a nonexponential plateau phase. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds, however, reaction components are consumed, and ultimately one or more of the components becomes limiting. At this point, the reaction slows and enters the plateau phase (cycles 28–40 in Figure 1.1).

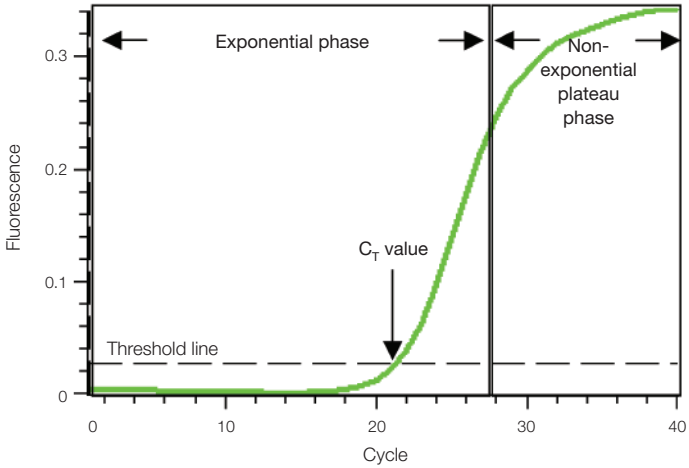



**Fig. 1.1. Amplification plot.** Baseline-subtracted fluorescence is shown.

Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable (cycles 1–18 in Figure 1.1) even though product accumulates exponentially. Eventually, enough amplified product accumulates to yield a detectable fluorescent signal. The cycle number at which this occurs is called the threshold cycle, or $C_T$. Since the $C_T$ value is measured in the exponential phase when reagents are not limited, real-time qPCR can be used to reliably and accurately calculate the initial amount of template present in the reaction.

The $C_T$ of a reaction is determined mainly by the amount of template present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background. Thus, the reaction will have a low, or early, $C_T$. In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background. Thus, the reaction will have a high, or late, $C_T$. This relationship forms the basis for the quantitative aspect of real-time PCR. Details of how $C_T$ values are used to quantify a sample are presented in Section 4.

### 1.1.3 Hallmarks of an Optimized qPCR Assay

Since real-time quantification is based on the relationship between initial template amount and the $C_T$ value obtained during amplification, an optimal qPCR assay is absolutely essential for accurate and reproducible quantification of your sample. The hallmarks of an optimized qPCR assay are:

- Linear standard curve ($R^2 > 0.980$ or $r > |–0.990|$)
- High amplification efficiency (90–105%)
- Consistency across replicate reactions

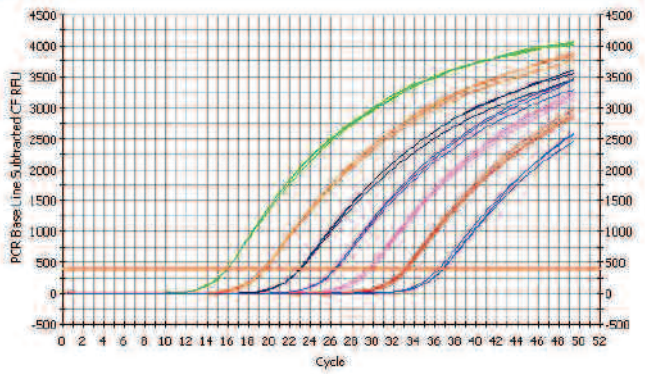
A powerful way to determine whether your qPCR assay is optimized is to run serial dilutions of a template and use the results to generate a standard curve. The template used for this purpose can be a target with known concentration (e.g., nanograms of genomic DNA or copies of plasmid DNA) or a sample of unknown quantity (e.g., cDNA). The standard curve is constructed by plotting the log of the starting quantity of template (or the dilution factor, for unknown quantities) against the $C_T$ value obtained during amplification of each dilution. The equation of the linear regression line, along with Pearson's correlation coefficient (r) or the coefficient of determination ($R^2$), can then be used to evaluate whether your qPCR assay is optimized.

Ideally, the dilution series will produce amplification curves that are evenly spaced, as shown in Figure 1.2 A. If perfect doubling occurs with each amplification cycle, the spacing of the fluorescence curves will be determined by the equation $2^n$ = dilution factor, where n is the number of cycles between curves at the fluorescence threshold (in other words, the difference between the $C_T$ values of the curves). For example, with a 10-fold serial dilution of DNA, $2^n = 10$. Therefore, n = 3.32, and the $C_T$ values should be separated by 3.32 cycles. Evenly spaced amplification curves will produce a linear standard curve, as shown in Figure 1.2 B. The equation and r value of the linear regression line are shown above the plot.

The r or $R^2$ value of a standard curve represents how well the experimental data fit the regression line, that is, how linear the data are. Linearity, in turn, gives a measure of the variability across assay replicates and whether the amplification efficiency is the same for different starting template copy numbers. A significant difference in observed $C_T$ values between replicates will lower the r or $R^2$ value. You should strive for an r whose absolute value is >0.990 or an $R^2$ value >0.980 for your qPCR reactions.

A




B

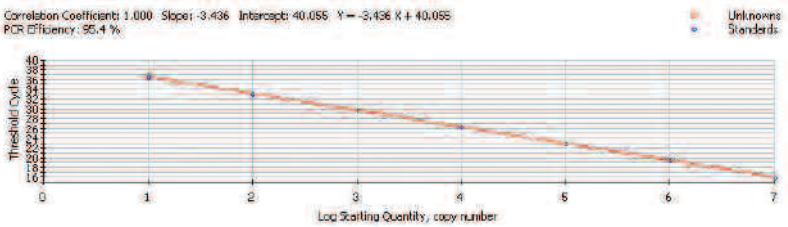



**Fig. 1.2. Generating a standard curve to assess reaction optimization.** A standard curve was generated using a 10-fold dilution of a template amplified on the iCycler iQ® real-time system. Each dilution was assayed in triplicate. **A**, Amplification curves of the dilution series. **B**, Standard curve with the $C_T$ plotted against the log of the starting quantity of template for each dilution. The equation for the regression line and the r value are shown above the graph. The calculated amplification efficiency was 95.4%.

Amplification efficiency, E, is calculated from the slope of the standard curve using the following formula:

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

Ideally, the amount of PCR product will perfectly double during each cycle of exponential amplification; that is, there will be a 2-fold increase in the number of copies with each cycle. This translates to a reaction efficiency of 2. Using an efficiency equal to 2 in the equation above, $2 = 10^{-1/slope}$, indicates that the optimal slope of the standard curve will be –3.32. Note that the absolute value of the slope is the same as the ideal spacing of the fluorescent traces described above.

Amplification efficiency is also frequently presented as a percentage, that is, the percent of template that was amplified in each cycle. To convert E into a percentage:

$$\% \text{ Efficiency} = (E - 1) \times 100\%$$

For an ideal reaction, % Efficiency = (2 – 1) x 100% = 100%.

For the example shown in Figure 1.2:

$$E = 10^{-(1/-3.436)} = 1.954$$

$$\% \text{ Efficiency} = (1.954 - 1) \times 100\% = 95.4\%$$

At the end of each cycle, the amplicon copy number increased 1.954-fold, or 95.4% of the template was amplified.

An efficiency close to 100% is the best indicator of a robust, reproducible assay. In practice, you should strive for an amplification efficiency of 90–105%. Low reaction efficiencies may be caused by poor primer design or by suboptimal reaction conditions. Reaction efficiencies >100% may indicate pipetting error in your serial dilutions or coamplification of nonspecific products, such as primer-dimers. When using the method described above to determine amplification efficiency, the presence of inhibitor can also result in an apparent increase in efficiency. This is because samples with the highest concentration of template also have the highest level of inhibitors, which cause a delayed $C_T$, whereas samples with lower template concentrations have lower levels of inhibitors, so the $C_T$ is minimally delayed. As a result, the absolute value of the slope decreases and the calculated efficiency appears to increase. If your reaction efficiency is <90% or >105%, we suggest that you modify your assay by redesigning your primers and probes. See Sections 2.3 and 2.4 for primer and probe design guidelines.

# Getting Started

# 2. Getting Started

Now that we have introduced the concepts behind real-time qPCR, we can delve into more practical matters. In this chapter, we offer recommendations for setting up your real-time qPCR assays, including basic laboratory techniques (Section 2.1), basic experimental design and reaction components (Section 2.2), deciding between singleplex vs. multiplex assays (Section 2.2.1), and selecting a real-time chemistry (Section 2.2.2). We also provide more detailed instructions for setting up qPCR reactions using the two most popular chemistries, SYBR Green I (Section 2.3) and TaqMan (Section 2.4).

## 2.1 General Considerations

Proper laboratory technique is an important yet often overlooked step toward successful real-time PCR. For optimal results, you should minimize the potential for cross-contamination between samples and prevent carryover of nucleic acids from one experiment to the next. The following precautions can help you avoid contamination problems:

- Wipe down workstations with dilute solutions of bleach or with commercially available decontamination solutions
- Prepare samples in a designated clean room, hood, or benchtop workstation equipped with a UV lamp. Ideally, this should be located in a different area from the thermal cycler. Take care to avoid contaminating the area with plasmids or amplicons; never bring postamplification products into the designated clean area
- Change gloves frequently during sample preparation and reaction setup
- Use shaftguard pipets and aerosol-barrier pipet tips
- Clean pipets frequently with a dilute solution of bleach
- Use PCR-grade water and reagents dedicated for PCR use only
- Use screw-capped tubes for dilutions and reaction setup

In addition to taking the above precautions, you should always prepare a no-template control for your PCR assays to verify that no contamination has occurred. Furthermore, use a hot-start polymerase to prevent indiscriminate amplification before the reaction begins.

To minimize statistical variation in experimental results, prepare master mixes for replicate reactions whenever possible. We recommend running all samples in triplicate. Minimize repeated freezing and thawing by preparing small aliquots of stock reagent before first use.

# 2.2 Experimental Design Considerations for qPCR

The first step in designing a real-time PCR experiment is to decide on the best type of assay for the experiment of interest. Should a singleplex or multiplex assay be used? What type of real-time chemistry should one employ? In the following sections, we describe each type of assay and provide guidelines on how to choose the best type of assay for your experiment.

## 2.2.1 Singleplex or Multiplex?

Both practical and scientific reasons might compel you to try multiplexing, the amplification of more than one target in a single reaction tube. Currently, it is possible to amplify and quantify as many as five targets in a single tube, depending on the features of your real-time PCR instrument. Multiplexing confers the following advantages over singleplex reactions:

- Reduction in the amount of starting template required, which is important when the amount of starting material is limited
- Reduction in false negatives, if a control target is amplified within each sample
- Increased laboratory throughput with a concomitant reduction in reagent costs
- Minimization of sample handling and associated opportunities for laboratory contamination

If none of these considerations are important for your assays, then singleplex reactions are sufficient. Practical advice on how to set up and optimize multiplex qPCR reactions is given in Section 3.

## 2.2.2 Chemistry Selection

A key step in designing a qPCR assay is selecting the chemistry to monitor the amplification of the target sequence. The variety of fluorescent chemistries available can be categorized into two major types: 1) DNA-binding dyes (SYBR Green I), and 2) dye-labeled, sequence-specific oligonucleotide primers or probes (molecular beacons and TaqMan, hybridization, and Eclipse probes, and Amplifluor, Scorpions, LUX, and BD QZyme primers). The most commonly used chemistries for real-time PCR are the DNA-binding dye SYBR Green I and TaqMan hydrolysis probes. We provide an overview of these different types of fluorescent chemistries in Sections 2.2.2.1–2.2.2.2.

The chemistry you select for your qPCR assay depends on your application, whether you're performing singleplex or multiplex reactions, and cost considerations. In general, for low-throughput, singleplex experiments, DNA-binding dyes may be preferable because these assays are easier to design, are faster to set up, and are initially more cost-effective. For high-throughput experiments, however, a fluorescent primer- or probe-based assay (singleplex or multiplex) may be more desirable because the initial cost can be spread over many experiments and the multiplex capability can reduce assay time. Multiplex assays require the use of a fluorescent primer- or probe-based chemistry, because the lack of specificity of DNA-binding dyes makes them incompatible with quantitative multiplex assays.

### 2.2.2.1 DNA-Binding Dyes (SYBR Green I)

The most commonly used DNA-binding dye for real-time PCR is SYBR Green I, which binds nonspecifically to double-stranded DNA (dsDNA). SYBR Green I exhibits little fluorescence when it is free in solution, but its fluorescence increases up to 1,000-fold when it binds dsDNA (Figure 2.1). Therefore, the overall fluorescent signal from a reaction is proportional to the amount of dsDNA present, and will increase as the target is amplified.

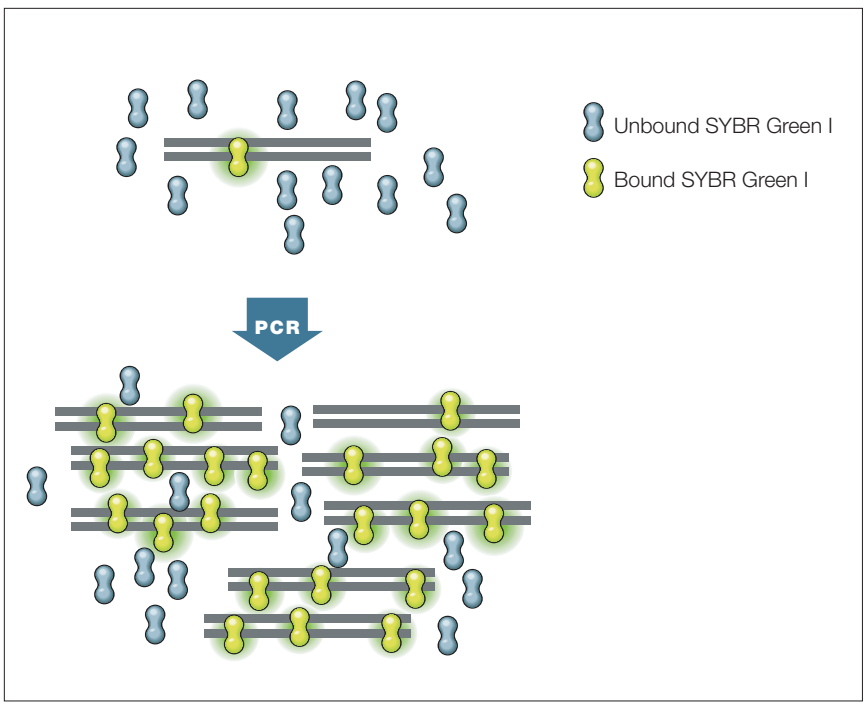



**Fig. 2.1. DNA-binding dyes in real-time PCR.** Fluorescence dramatically increases when the dye molecules bind to dsDNA.

The advantages of using dsDNA-binding dyes include simple assay design (only two primers are needed; probe design is not necessary), ability to test multiple genes quickly without designing multiple probes (e.g., for validation of gene expression data from many genes in a microarray experiment), lower initial cost (probes cost more), and the ability to perform a melt-curve analysis to check the specificity of the amplification reaction.

Melt-curve analysis can be used to identify different reaction products, including nonspecific products. After completion of the amplification reaction, a melt curve is generated by increasing the temperature in small increments and monitoring the fluorescent signal at each step. As the dsDNA in the reaction denatures (i.e., as the DNA "melts"), the fluorescence decreases. The negative first derivative of the change in fluorescence is plotted as a function of temperature. A characteristic peak at the amplicon's melting temperature ($T_m$, the temperature at which 50% of the base pairs of a DNA duplex are separated) distinguishes it from other products such as primer-dimers, which melt at different temperatures. An example of this is shown in Figure 2.2. The melt peak with a $T_m$ of 89°C represents the specific product, and corresponds to the upper band in lanes 2 and 3 on the gel. The peak with a $T_m$ of 78°C represents the nonspecific product, and corresponds to the lower band in lanes 2 and 3 on the gel.

The major drawback of DNA-binding dyes is their lack of specificity, that is, DNA-binding dyes bind to any dsDNA. As a result, the presence of nonspecific products in a real-time PCR reaction may contribute to the overall fluorescence and affect the accuracy of quantification. Another consequence is that DNA-binding dyes cannot be used for multiplex reactions because fluorescent signals from different amplicons cannot be distinguished. Instead, you can set up parallel reactions to examine multiple genes, such as a gene of interest and reference gene, in a real-time PCR assay with SYBR Green I.

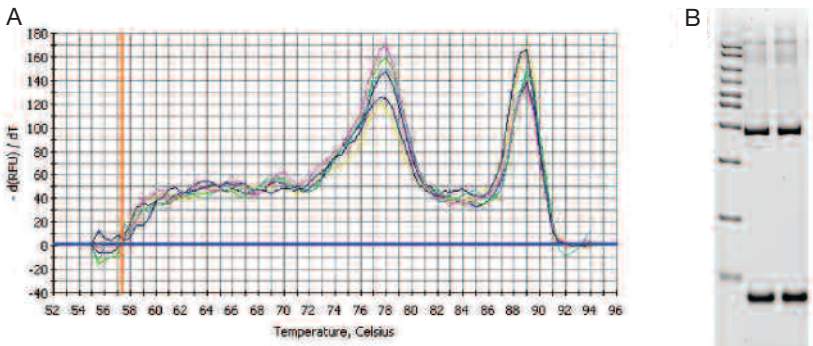



**Fig. 2.2. Melt-curve analysis of reaction product from a SYBR Green I assay.** The melt-curve analysis function of real-time instruments can be used to distinguish specific products from nonspecific products. **A**, The negative first derivative of the change in fluorescence is plotted as a function temperature. The two peaks indicate the $T_m$ values of two PCR products. **B**, Gel analysis of the qPCR products. Lane 1, AmpliSize® 50–2,000 base pairs (bp) molecular ruler; lanes 2 and 3, two replicates of qPCR product from the reaction shown in (A). The two PCR products are revealed by separate bands in the gel.

### 2.2.2.2 Fluorescent Primer- and Probe-Based Chemistries

Many fluorescent primer- and probe-based chemistries have been devised and are available from different commercial vendors. The most commonly used probe-based chemistries, TaqMan probes and molecular beacons, are discussed in detail below. Brief descriptions of other fluorescent primer- and probe-based chemistries, including Eclipse probes and Amplifluor, Scorpions, LUX, and BD QZyme primers, are also presented below.

Primer- and probe-based detection chemistries share some common features. In general, these chemistries take advantage of fluorescence resonance energy transfer (FRET), or some other form of fluorescence quenching, to ensure that specific fluorescence is detected only in the presence of amplified product. The primer or target-specific oligonucleotide probe is labeled with a reporter fluorophore; but in most cases, the oligonucleotide is designed so that fluorescence is quenched when the specific target is unavailable. Usually this is accomplished by attaching a quencher molecule to the probe, and devising some mechanism by which the reporter and quencher are separated when the probe binds to its specific target. The details of how each chemistry achieves this separation will be described in the following sections.

In real-time PCR, fluorescent primers and probes offer two main advantages over DNA-binding dyes. First, they specifically detect the target sequence so nonspecific products do not affect the accuracy of quantification. Second, they allow multiplex reactions to be performed.

**TaqMan Assays**

TaqMan assays employ a sequence-specific, fluorescently labeled oligonucleotide probe called the TaqMan probe, in addition to the sequence-specific primers. Figure 2.3 illustrates how TaqMan assays work. Also known as the 5'-nuclease assay, the TaqMan assay exploits the 5'-exonuclease activity of certain thermostable polymerases, such as *Taq* or *Tth*. The probe contains a fluorescent reporter at the 5' end and a quencher at the 3' end. When intact, the fluorescence of the reporter is quenched due to its proximity to the quencher. During the combined annealing/extension step of the amplification reaction, the probe hybridizes to the target and the dsDNA-specific 5'→3' exonuclease activity of *Taq* or *Tth* cleaves off the reporter. As a result, the reporter is separated from the quencher, and the resulting fluorescence signal is proportional to the amount of amplified product in the sample. One commonly used fluorescent reporter-quencher pair is fluorescein (FAM, which emits green fluorescence) and Black Hole Quencher 1. For more information about suitable reporter-quencher pairs, see Table 2.1.

The main advantages of using TaqMan probes include high specificity, a high signal-to-noise ratio, and the ability to perform multiplex reactions. The disadvantages are that the initial cost of the probe may be high and the assay design may not be trivial. See Section 2.4.1 for details on TaqMan assay design.

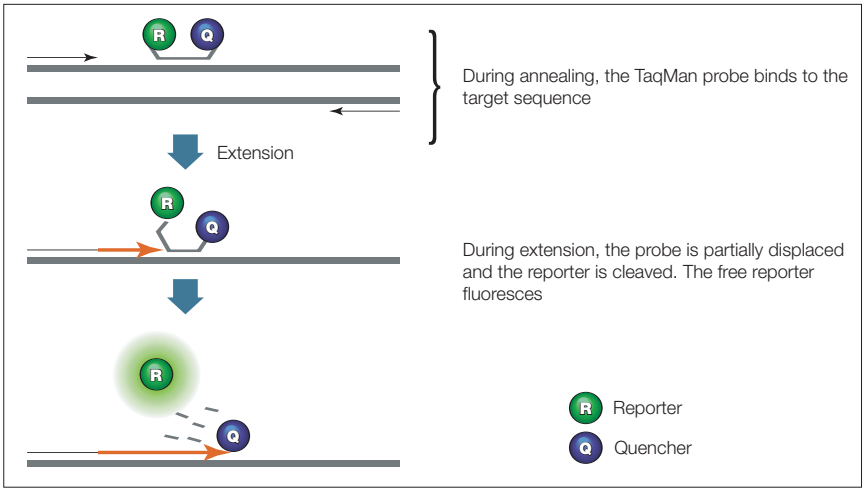



**Fig. 2.3. TaqMan assay.**

### Molecular Beacons

In addition to two sequence-specific primers, molecular beacon assays employ a sequence-specific, fluorescently labeled oligonucleotide probe called a molecular beacon, which is a dye-labeled oligonucleotide (25–40 nt) that forms a hairpin structure with a stem and a loop (Figure 2.4). A fluorescent reporter is attached to the 5' end of the molecular beacon and a quencher is attached to the 3' end. The loop is designed to hybridize specifically to a 15–30 nucleotide section of the target sequence. On either side of the loop are 5–6 nt that are complementary to each other, and form a stem structure that serves to bring the reporter and quencher together. In the hairpin structure, no fluorescence is detected from the reporter due to its physical proximity to the quencher. During the annealing step of the amplification reaction, the molecular beacon binds to its target sequence, separating the reporter and quencher such that the reporter is no longer quenched. Unlike TaqMan assays, molecular beacons are displaced but not destroyed during amplification, because a DNA polymerase lacking the 5' exonuclease activity is used. The amount of fluorescence emitted by the reporter on the molecular beacon is proportional to the amount of target in the reaction.

Molecular beacons have some advantages over other chemistries. They are highly specific, can be used for multiplexing, and if the target sequence does not match the beacon sequence exactly, hybridization and fluorescence will not occur — a desirable quality for allelic discrimination experiments.

The main disadvantage of using molecular beacons lies in their design. The stem of the hairpin must be strong enough that the molecule will not spontaneously fold into non-hairpin conformations that result in unintended fluorescence. At the same time, the stem of the hairpin must not be too strong, or the beacon may not properly hybridize to the target.

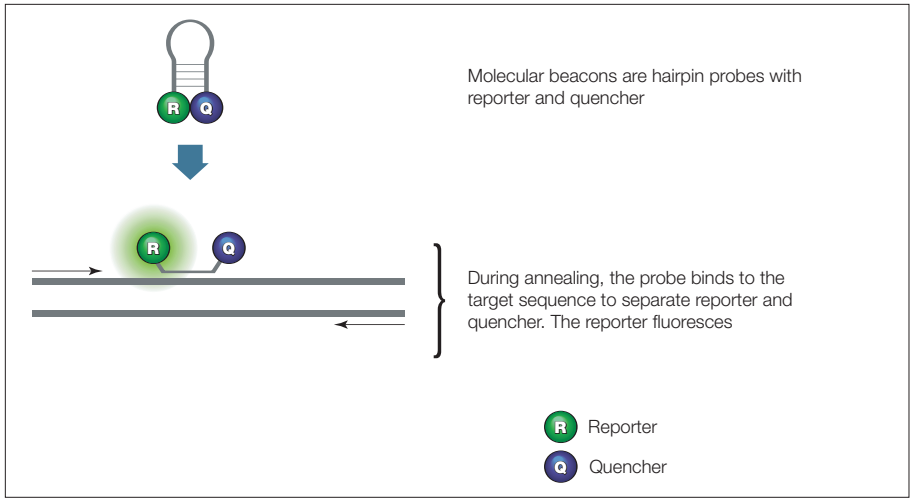



Fig. 2.4. Molecular beacons.

### Other Chemistries

**Hybridization probe** assays employ two sequence-specific oligonucleotide probes, in addition to two sequence-specific primers. The two probes are designed to bind to adjacent sequences in the target, as illustrated in Figure 2.5. The first probe carries a donor dye at its 3' end, while the second carries an acceptor dye at its 5' end. The donor and the acceptor dyes are selected such that the emission spectrum of the donor dye overlaps significantly with the excitation spectrum of the acceptor dye, while the emission spectrum of the donor dye is spectrally separated from the emission spectrum of the acceptor dye. Excitation is performed at a wavelength specific to the donor dye, and the reaction is monitored at the emission wavelength of the acceptor dye. During the annealing step of PCR, the probes hybridize to their target sequences in a head-to-tail arrangement. This brings the fluorescent molecules into proximity, allowing fluorescence resonance energy transfer from donor to acceptor. The increasing amount of acceptor fluorescence is proportional to the amount of amplicon present.

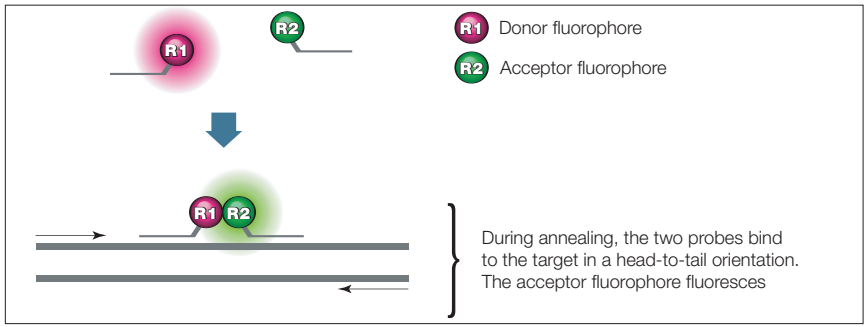



Fig. 2.5. Hybridization probes.

qPCR assays using an **Eclipse probe** employ two primers and a sequence-specific oligonucleotide probe, as illustrated in Figure 2.6. The probe is complementary to a sequence within the amplicon and contains a fluorescent reporter at the 3' end, a quencher at the 5' end, and a minor groove binder. The unhybridized probe adopts a conformation that brings the reporter and quencher together, quenching the reporter. During the annealing step of PCR, the probe hybridizes to the target with the help of the minor groove binder. The probe thus becomes linearized, separating the reporter and quencher and allowing the reporter to fluoresce. The resulting fluorescent signal is proportional to the amount of amplified product in the sample.

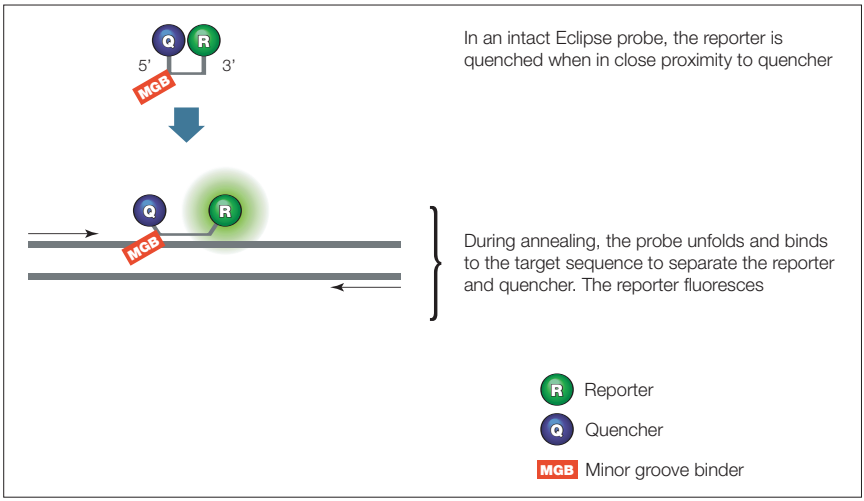



**Fig. 2.6. Eclipse probes.**

qPCR assays using **Amplifluor chemistry** employ two target-specific primers and one universal primer called the UniPrimer. The first target-specific primer contains a 5' extension sequence called the Z-sequence that is also found at the 3' end of the UniPrimer. As shown in Figure 2.7, the UniPrimer forms a hairpin structure. A fluorescent reporter and a quencher are attached at the 5' and the 3' ends of the stem structure, respectively. In the hairpin conformation, the reporter fluorescence is quenched due to its proximity to the quencher. During the first amplification cycle, the first target-specific primer (with the Z-sequence) hybridizes to the template and is extended. During the second amplification cycle, the second target-specific primer is used to synthesize a new target template that contains a sequence complementary to the Z-sequence. The product from the second amplification cycle can then serve as the template for the UniPrimer. In the third amplification cycle, the extended UniPrimer serves as a template for the next amplification cycle. In the fourth cycle, extension of the template through the hairpin region of the UniPrimer causes the UniPrimer to open up and adopt a linear configuration,

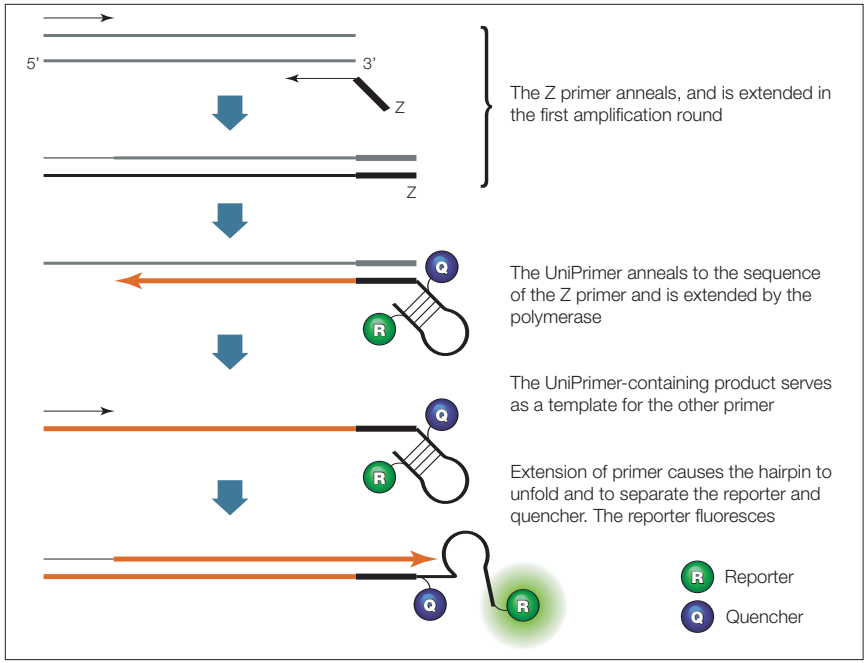



**Fig. 2.7. Amplifluor primer.**

which allows the reporter to fluoresce. Exponential amplification using the second target-specific primer and the UniPrimer occurs in subsequent amplification cycles. The resulting fluorescent signal is proportional to the amount of amplified product in the sample.

**Scorpions primer** assays employ two primers, one of which serves as a probe and contains a stem-loop structure with a 5' fluorescent reporter and 3' quencher, as illustrated in Figure 2.8. The loop sequence of the Scorpions probe is complementary to an internal portion of the target sequence on the same strand. During the first amplification cycle, the Scorpions primer is extended and the sequence complementary to the loop sequence is generated on the same strand. The Scorpions probe contains a PCR blocker just 3' of the quencher to prevent read-through during the extension of the opposite strand. After subsequent denaturation and annealing, the loop of the Scorpions probe hybridizes to the target sequence by an intramolecular interaction, and the reporter is separated from the quencher. The resulting fluorescent signal is proportional to the amount of amplified product in the sample.

**LUX** primer assays employ two primers, one of which is a hairpin-shaped primer with a fluorescent reporter attached near the 3' end, as illustrated in Figure 2.9. In the intact primer, the reporter is quenched by the secondary structure of the hairpin. During amplification, the LUX primer is incorporated into the product and the reporter fluoresces.

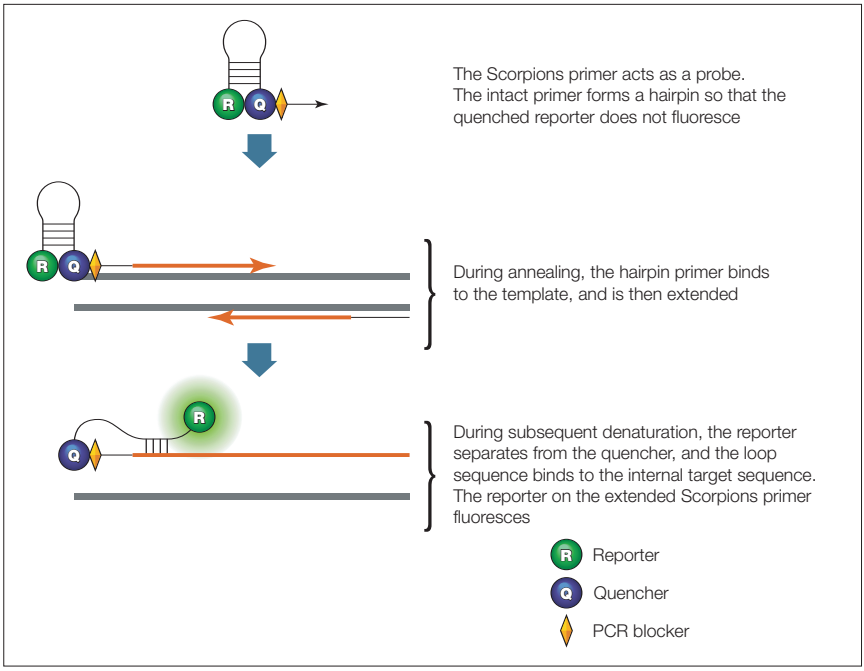



**Fig. 2.8. Scorpions primer.**

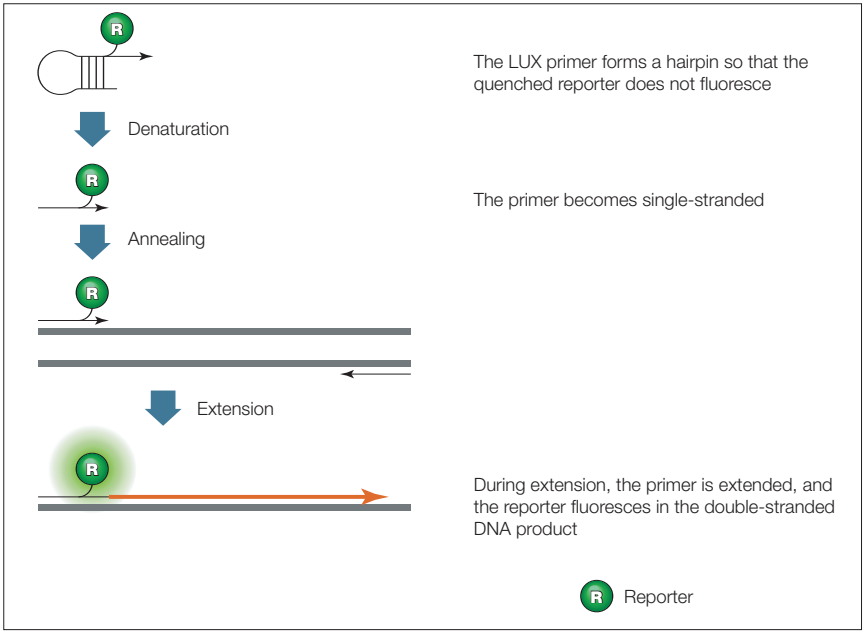



**Fig. 2.9. LUX primers.**

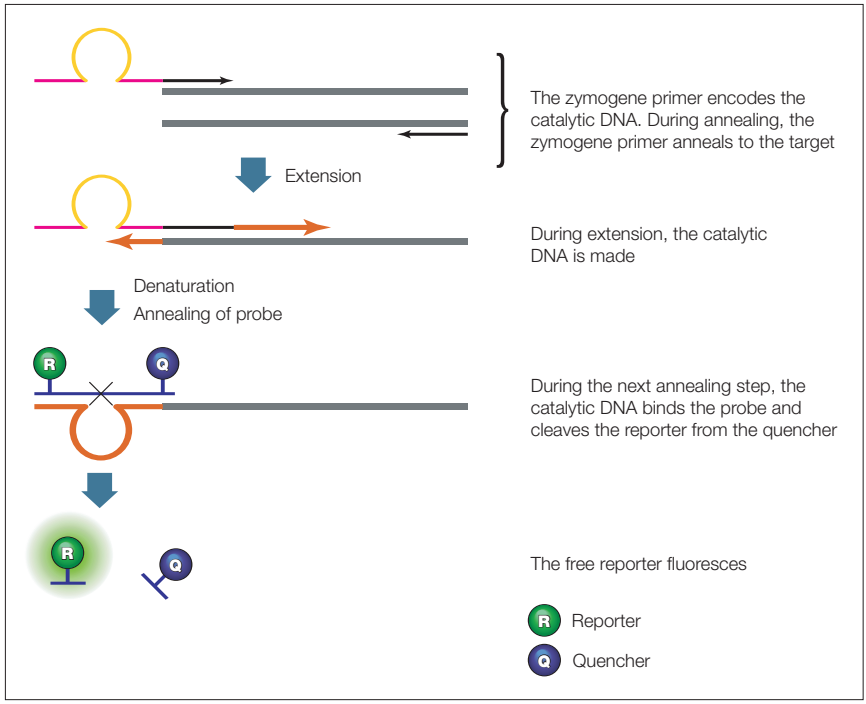



**Fig. 2.10. BD QZyme primers.**

qPCR assays using **BD QZyme** primers employ a target-specific zymogene primer, a target-specific reverse primer, and a universal oligonucleotide substrate as shown in Figure 2.10. The oligonucleotide contains a fluorescent reporter on the 5' end and a quencher on the 3' end. When oligonucleotide substrate is intact, the fluorescence of the reporter is quenched by the quencher due to their proximity. The zymogene primer contains a sequence that encodes a catalytic DNA. During the first amplification cycle, the zymogene primer is extended. In the second cycle, the product of the first cycle is used as the template by the target-specific reverse primer, which is extended to create a new target sequence containing a catalytic DNA region. In the subsequent annealing step, the fluorescently labeled oligonucleotide substrate hybridizes to the catalytic DNA sequence and is cleaved. This cleavage separates the reporter from the quencher, resulting in a fluorescent signal that is proportional to the amount of amplified product in the sample.

# 2.3 Design and Optimization of SYBR Green I Reactions

A SYBR Green I assay uses a pair of PCR primers that amplifies a specific region within the target sequence of interest and includes SYBR Green 1 for detecting the amplified product. The steps for developing a SYBR Green I assay are:

- Primer design and amplicon design
- Assay validation and optimization

Sections 2.3.1 and 2.3.2 provide guidelines on how to carry out these two steps.

## 2.3.1 Primer and Amplicon Design

A successful real-time PCR reaction requires efficient and specific amplification of the product. Both primers and target sequence can affect this efficiency. Therefore, care must be taken when choosing a target sequence and designing primers. A number of free and commercially available software programs are available for this purpose. One popular web-based program for primer design is Primer3 (**http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi**). A commercially available program such as Beacon Designer software performs both primer design and amplicon selection.

For amplicon design, follow these guidelines:

- Design amplicon to be 75–200 bp. Shorter amplicons are typically amplified with higher efficiency. An amplicon should be at least 75 bp to easily distinguish it from any primer-dimers that might form
- Avoid secondary structure if possible. Use programs such as mfold (**http://www.bioinfo.rpi.edu/applications/mfold/**) to predict whether an amplicon will form any secondary structure at annealing temperature. See Real-Time PCR: General Considerations (Bio-Rad bulletin 2593) for more details
- Avoid templates with long (>4) repeats of single bases
- Maintain a GC content of 50–60%

For primer design, follow these parameters:

- Design primers with a GC content of 50–60%
- Maintain a melting temperature ($T_m$) between 50ºC and 65ºC. We calculate $T_m$ values using the nearest-neighbor method with values of 50 mM for salt concentration and 300 nM for oligonucleotide concentration
- Avoid secondary structure; adjust primer locations outside of the target sequence secondary structure if required

- Avoid repeats of Gs or Cs longer than three bases
- Place Gs and Cs on ends of primers
- Check sequence of forward and reverse primers to ensure no 3' complementarity (avoid primer-dimer formation)
- Verify specificity using tools such as the Basic Local Alignment Search Tool (**http://www.ncbi.nlm.nih.gov/blast/**)

## 2.3.2 Assay Validation and Optimization

A SYBR Green I qPCR reaction contains the following components:

- PCR master mix with SYBR Green I
- Template
- Primers

Preformulated real-time PCR master mixes containing buffer, DNA polymerase, dNTPs, and SYBR Green I dye are available from several vendors. We recommend iQ™ SYBR® Green supermix.

Optimized SYBR Green I qPCR reactions should be sensitive and specific and should exhibit good amplification efficiency over a broad dynamic range. To determine the performance of your SYBR Green I qPCR assay, perform the following two steps:

- Identify the optimal annealing temperature for your assay
- Construct a standard curve to evaluate assay performance

### 2.3.2.1 Annealing Temperature Optimization

The optimal annealing temperature can easily be assessed on qPCR instruments that have a temperature gradient feature, such as the MiniOpticon™, MyiQ™, DNA Engine Opticon®, Opticon™ 2, iCycler iQ®, Chromo4™, and iQ™5 systems. A gradient feature allows you to test a range of annealing temperatures simultaneously, so optimization reactions can be performed in a single experiment.

To find the optimal annealing temperature for your reaction, we recommend testing a range of annealing temperatures above and below the calculated $T_m$ of the primers. A sample annealing temperature optimization experiment is shown in Figure 2.11.

Because SYBR Green I binds to all dsDNA, it is necessary to check the specificity of your qPCR assay by analyzing the reaction product(s). To do this, use the melt-curve function on your real-time instrument and also run products on an agarose gel. Figure 2.12 shows the melt-curve and agarose gel analyses from the annealing temperature optimization experiment shown in Figure 2.11. The reaction with annealing temperature at 62.2°C is shown. An optimized SYBR Green I qPCR reaction should have a single peak in the melt curve, corresponding to the single band on the agarose gel, as shown in Figure 2.12.

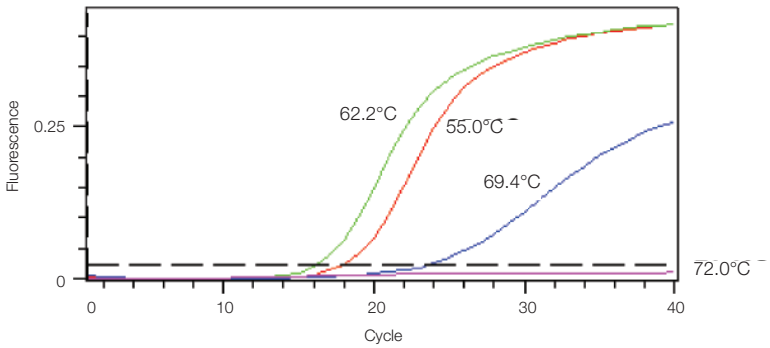



**Fig. 2.11. Annealing temperature optimization.** An annealing temperature gradient of 55°C to 72°C was performed on the Chromo4 system. The 62.2°C reaction gave the lowest $C_T$ value and was selected as the optimal annealing temperature for this assay.

Nonspecific products that may have been coamplified with the specific product can be identified by melt-curve analysis (Figure 2.13). In this example, the specific product is the peak with a $T_m$ of 89°C and corresponds to the upper band on the gel. The nonspecific product is the peak with a $T_m$ of 78°C and corresponds to the lower band in the gel. By comparing the gel image with the melt curve, you can identify peaks in the melt curve that correspond to specific product, additional nonspecific bands, and primer-dimers. If nonspecific products such as primer-dimers are detected by melt-curve analysis, we recommend that you redesign your primers (see Section 2.3.1).

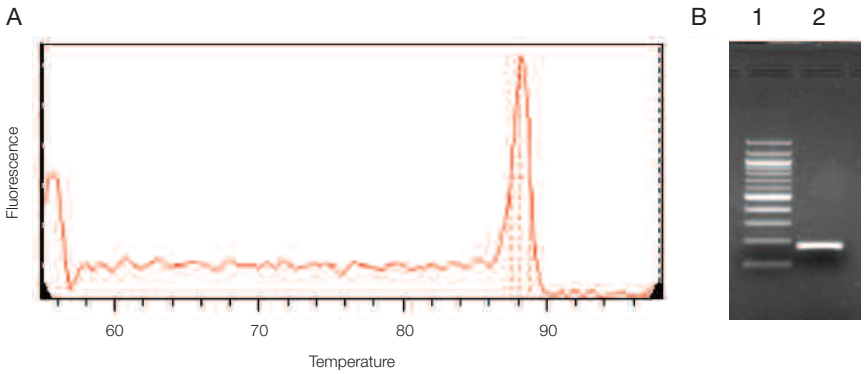



**Fig. 2.12. Validation of SYBR Green I reactions. A**, melt-curve analysis, with the first derivative of the change in fluorescence intensity as a function temperature plotted, and **B**, agarose gel analysis of the reaction products. Lane 1, molecular weight markers, 100–1,000 bp in 100 bp increments, 1,200 and 1,500 bp; lane 2, single PCR product corresponding to peak observed in (A).

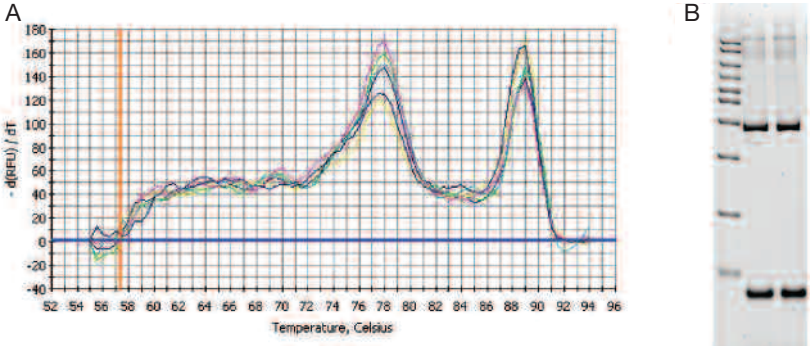



**Fig. 2.13. Melt-curve analysis.** The presence of a nonspecific product, in this case primer-dimers, shows up as an additional peak in the melt-curve analysis. **A**, The negative first derivative of the change in fluorescence is plotted as a function of temperature. **B**, Gel analysis of the qPCR products. Lane 1, AmpliSize 50–2,000 bp molecular ruler; lanes 2 and 3, two replicates from the reaction shown in (A).

### 2.3.2.2 Assay Performance Evaluation Using Standard Curves

The efficiency, reproducibility, and dynamic range of a SYBR Green I assay can be determined by constructing a standard curve using serial dilutions of a known template, as described in Section 1.1.3. The efficiency of the assay should be 90–105%, the $R^2$ of the standard curve should be >0.980 or r > |–0.990|, and the $C_T$ values of the replicates should be similar.

It is important to note that the range of template concentrations used for the standard curve must encompass the entire range of template concentration of the test samples to show that results from the test samples are within the linear dynamic range of the assay. If the test samples give results outside of the range of the standard curves, one of the following must be performed:

- Construct a wider standard curve covering the test sample concentrations and perform analysis to ensure that the assay is linear in that new range
- If the test samples give a lower $C_T$ than the highest concentration of standards used in the standard curve, repeat the assay using diluted test samples
- If the test samples give a higher $C_T$ than the lowest concentration of standards used in the standard curve, repeat the assay using larger amounts of the test samples

# 2.4 Design and Optimization of TaqMan Probe Reactions

A TaqMan assay uses a pair of PCR primers and a dual-labeled target-specific fluorescent probe. The steps for developing a TaqMan assay are:

- Primer and probe design
- Assay validation and optimization

Sections 2.4.1 and 2.4.2 provide guidelines on how to carry out these two steps.

## 2.4.1 Primer and Probe Design

As with any qPCR reaction, TaqMan-based assays require efficient and specific amplification of the product. Primer design rules are similar to those described in Section 2.3.1. Typically, the primers are designed to have an annealing temperature between 55 and 60ºC. We recommend using software such as Beacon Designer for designing your TaqMan primers and TaqMan probe. Because the dual-labeled probe is the most costly component of a TaqMan assay, we suggest that you order the two primers and validate their performance using SYBR Green I before ordering the dual-labeled probe. See Section 2.3 for details on how to validate a primer set in a SYBR Green I reaction.

The TaqMan probe should have a $T_m$ 5–10°C higher than that of the primers. In most cases, the probe should be <30 nucleotides and must not contain a G at its 5' end because this could quench the fluorescent signal even after hydrolysis. Choose a sequence within the target that has a GC content of 30–80%, and design the probe to anneal to the strand that has more Gs than Cs (so the probe contains more Cs than Gs).

An important aspect of designing a TaqMan probe is reporter and quencher selection. A list of commonly used combinations of reporters and quenchers is shown in Table 2.1. We recommend using FAM-labeled probes when designing singleplex reactions, because they are inexpensive and readily available, perform well, and can be detected by all instruments currently on the market.

**Table 2.1. Commonly used combinations of reporters and quenchers.**

| Reporter | Compatible Quencher |
|---|---|
| FAM | BHQ1, DABCYL,* TAMRA** |
| TET | BHQ1, DABCYL* |
| HEX | BHQ1, BHQ2, DABCYL* |
| TAMRA | BHQ2, DABCYL* |
| Texas Red | BHQ2, BHQ3, DABCYL* |
| ROX | BHQ2, BHQ3, DABCYL* |
| Cy5 | BHQ2, BHQ3 |

* DABCYL is a mechanical quencher only appropriate for molecular beacons.

** TAMRA is not recommended as a quencher, but will work for single-color FAM assays. TAMRA will not work well as a quencher on molecular beacons.

Another important consideration for obtaining accurate real-time qPCR data is probe quality. Even a perfectly designed probe can fail if the probe is improperly synthesized or purified. Improper removal of uncoupled fluorescent label, inefficient coupling, and/or poor quenching can produce high fluorescent background or noise. A low signal-to-noise ratio results in decreased sensitivity and a smaller linear dynamic range. Two probes with identical sequences and identical fluorophore labels can be measurably different when synthesized by different suppliers or even at different times by the same supplier. We recommend saving aliquots of each probe for direct comparisons with newly synthesized probes.

## 2.4.2 Assay Validation and Optimization

A TaqMan probe-based qPCR reaction contains the following components:

- PCR master mix
- Template
- Primers
- Probe(s)

Preformulated PCR master mixes containing buffer, DNA polymerase, and dNTPs are commercially available from several vendors. For TaqMan assays, we recommend using iQ™ supermix with 300 nM of each of the two primers and 200 nM of probe(s).

TaqMan assays require careful attention to temperature conditions. A typical TaqMan protocol contains a denaturation step followed by a combined annealing and extension step at 55–60°C, instead of the traditional three-step PCR cycle of denaturation, annealing, and extension. This is to ensure that the probe remains bound to its target during primer extension. Typical TaqMan probes for nucleic acid quantification are designed to have a $T_m$ of 60–70°C.

An optimized TaqMan assay should be sensitive and specific, and should exhibit good amplification efficiency over a broad dynamic range. To determine the performance of your TaqMan assay, use the procedure outlined in Sections 1.1.3 and 2.3.2. In short, construct a standard curve using dilutions of a known template and use this curve to determine the efficiency of the assay along with $R^2$ or r of the regression line. The efficiency of the reaction should be between 90 and 105%, the $R^2$ should be >0.980 or r > |–0.990|, and the replicates should give similar $C_T$ values.

If the assay performs within these specifications, you are ready to start your experiment. If the assay performs outside these specifications, we suggest that you redesign your primers and TaqMan probe.

It is important to note that the range of template concentrations used for the standard curve must encompass the entire range of template concentrations of the test samples to demonstrate that results from the test samples are within the dynamic range of the assay. If test samples give results outside the range of the standard curve, one of the three following steps must be performed:

- Construct a wider standard curve covering the test sample concentrations and perform analysis to ensure that the assay is linear in that new range
- If the test samples give a lower $C_T$ than the highest concentration of standards used in the standard curve, repeat the assay using diluted test samples
- If the test samples give a higher $C_T$ than the lowest concentration of standards used in the standard curve, repeat the assay using larger amounts of the test samples

# Multiplexing Considerations

# 3. Multiplexing Considerations

If you have decided to perform your qPCR experiments by amplifying more than one target in a single reaction tube, that is, multiplexing (see Section 2.2.1), read the following section before beginning your experiments.

Successful multiplexing results from careful experimental design and optimization of reaction conditions, not from simply combining all primers and templates in the same tube. This is because amplification of any one target can influence the amplification of the other targets in the same tube.

A common challenge for multiplex real-time PCR is amplifying targets that have significantly different concentrations. An example comes from gene expression studies in which a gene of interest (GOI) is compared to a reference gene. If the reference gene is present at great excess and has a higher amplification efficiency than the GOI, the amplification of the GOI may be outcompeted and thus compromised by amplification of the reference gene when both are amplified in the same tube (multiplexed). This would result in an underestimation of the amount of GOI present in the sample.

The following steps should be used to develop a multiplex assay:

- Design primer and probe sequences
- Select reporters and quenchers for probes
- Optimize individual assays
- Validate the multiplex assay
- Optimize the multiplex assay, if necessary

In the following sections, we discuss each of these steps in detail.

## 3.1 Primer and Probe Design for Multiplexing

If you are setting up a multiplex TaqMan assay, follow the guidelines in Section 2.4 to design the individual TaqMan assays. Design all primers with approximately the same $T_m$ (55–60°C), and all probes with approximately the same $T_m$ (~5–10°C higher than the primers). It is important to make sure that the different primer and probe sets do not exhibit complementarity to one another because all primers and probes will be present in one reaction. We recommend the use of commercially available software such as Beacon Designer to simplify the design of primer and probe sets for multiplex reactions.

## 3.2 Selection of Reporters and Quenchers for Multiplexing

Multiplex reactions require the use of multiple reporters to follow each individual amplification reaction. To distinguish each reaction, choose reporter fluorophores with minimally overlapping emission spectra. The selected fluorophores also need to be compatible with the excitation and emission filters of your real-time instrument. See your instrument manual for a list of compatible fluorophores. For example, for a fourplex reaction on the Chromo4™, iCycler iQ®, and iQ™5 instruments, we recommend using FAM, HEX, Texas Red, and Cy5. For a fiveplex assay on the iQ5 system, we recommend using FAM, HEX, TAMRA, Texas Red, and Cy5.

## 3.3 Optimization of Individual Assays Before Multiplexing

The first step in assembling a multiplex assay is to optimize the individual reactions. Determine the efficiency of each individual reaction by constructing a standard curve using a series of template dilutions as described in Sections 1.1.3 and 2.3.2. The efficiency of the individual reactions should be 90–105%.

## 3.4 Validation of Multiplex Assays

Run singleplex and multiplex assays for your targets on the same plate and compare the $C_T$ values. The values obtained for a given target in the singleplex and multiplex assays should not be significantly different. If the $C_T$ values from the singleplex and the multiplex reaction are significantly different, you will need to optimize your reactions. This can be achieved by varying the concentration of reaction components as described in Section 3.5.

iQ™ multiplex powermix greatly simplifies optimization of multiplex assays, routinely yielding similar efficiencies in singleplex and multiplex assays.

# 3.5 Optimization of Multiplex Assays

If a multiplex reaction is not optimized, the amplification of the less-efficient or less-abundant target can be inhibited by the more-efficient or more-abundant target. This is because reaction components, such as DNA polymerase and nucleotides, become limiting in later cycles and the amplification of the less-efficient or less-abundant target is compromised.

This inhibitory effect is apparent when comparing the amplification plot of a fixed amount of amplicon in a singleplex reaction to that of the same amplicon in a multiplex reaction: the $C_T$ for the multiplex reaction is delayed compared to the $C_T$ for the singleplex reaction. This is demonstrated in Figure 3.1, where amplification of four targets, β-actin, ornithine decarboxylase (ODC), ODC antizyme (OAZ), and antizyme inhibitor (AZI), was carried out in singleplex reactions (dark blue line) and in a multiplex reaction with otherwise identical reaction components (light blue line). Amplification of the two less-abundant targets, ODC and AZI, was affected, and resulted in delayed $C_T$ values in the multiplex reactions compared to the corresponding singleplex reactions.

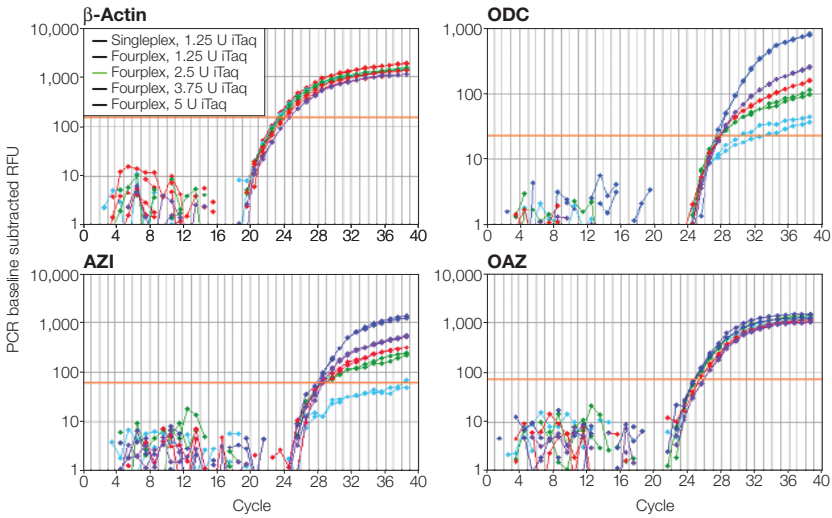



**Fig. 3.1. Optimization of iTaq™ DNA polymerase concentration in fourplex reactions.** $MgCl_2$ and dNTP concentrations were kept constant while the singleplex iTaq concentration (1x) was compared to multiplex conditions using increasing amounts of iTaq DNA polymerase (1–4x). The β-actin and OAZ genes (those with the highest expression in this example) were amplified with similar efficiency in singleplex and multiplex conditions at all iTaq concentrations tested. The genes with lower expression, AZI and ODC, required additional iTaq polymerase to attain multiplex amplification efficiencies comparable to those of the singleplex amplification.

One method for optimizing multiplex reactions is to increase the concentrations of DNA polymerase, dNTPs, and $MgCl_2$. To optimize the amplification of β-actin, ODC, AZI, and OAZ in the multiplex reaction, we sequentially increased the concentrations of DNA polymerase, $MgCl_2$, and dNTPs.

First, we increased the concentration of the iTaq DNA polymerase in the multiplex reaction, in 1.25 unit (U) increments, from 1.25 U per reaction to 5 U per reaction. With increased concentrations of DNA polymerase, we observed increased amplification efficiencies for ODC and AZI, as shown in Figure 3.1. Based on these data, we chose to use 3.75 U DNA polymerase in the subsequent optimization steps.

Next we tested the effect of increasing $MgCl_2$ from 3.5 mM to 5 mM, and dNTPs from 200 µM to 400 µM. As shown in Figure 3.2, when the iTaq DNA polymerase concentration was set at 3.75 U per 50 µl reaction, our four-gene multiplex assay benefited from 5 mM $MgCl_2$, with no increase necessary for dNTPs.

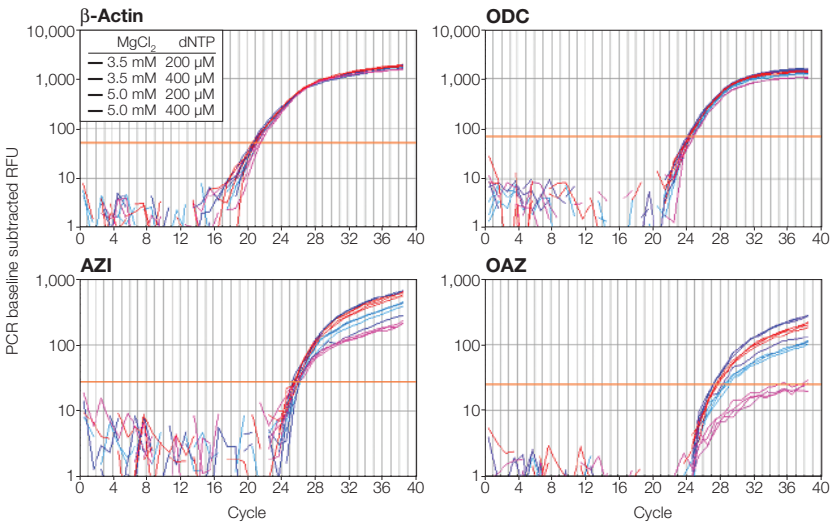
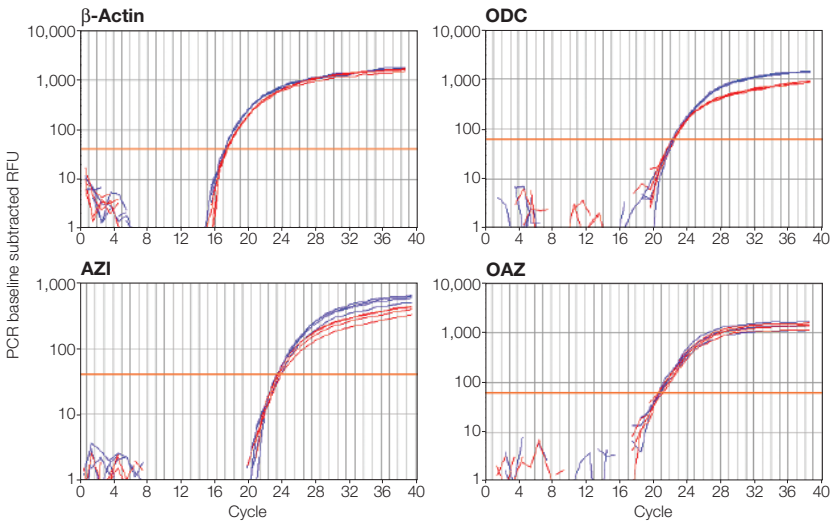



**Fig. 3.2. Optimization of $MgCl_2$ and dNTP concentrations in fourplex reactions.** While keeping the iTaq DNA polymerase concentration constant at 3.75 U per 50 µl reaction, the effects of $MgCl_2$ and dNTPs concentration were assessed. The combination of 5.0 mM $MgCl_2$, 200 µM dNTPs, and 3.75 U iTaq DNA polymerase gave the best amplification in multiplex reactions (no singleplex reactions are shown here).

To verify the performance of the optimized multiplex assay, we performed side-by-side comparisons of single- and multiplex reactions for each primer-probe set at the optimized condition (3.75 U iTaq, 5 mM $MgCl_2$, 200 µM dNTPs). The real-time amplification plots generated using singleplex and multiplex conditions should show the same $C_T$ values and be superimposable during the exponential phase of the amplification. Successful multiplex conditions were indeed achieved when using total RNA samples from human prostate (Figure 3.3) and small intestine (data not shown) as template.




| | $C_T$ Values | | | |
|---|---|---|---|---|
| | **β-Actin** | **ODC** | **AZI** | **OAZ** |
| Singleplex | 17.0 ± 0.0 | 23.0 ± 0.2 | 22.2 ± 0.1 | 20.8 ± 0.1 |
| Multiplex | 17.3 ± 0.0 | 23.1 ± 0.2 | 22.2 ± 0.1 | 20.8 ± 0.2 |

**Fig. 3.3. Verification of multiplex assay performance.** Data from singleplex (blue) and fourplex (red) reactions (four replicates, each using human prostate cDNA as template) are superimposed. The plots overlap completely during the exponential phase. The table shows the average $C_T$ values for the singleplex and multiplex reactions.

# Real-Time qPCR Data Analysis

# 4. Real-Time qPCR Data Analysis

As discussed in Section 1, real-time qPCR is a method for determining the amount of nucleic acid in a sample. Knowing that you have 500,000 molecules of p53 mRNA in your sample tube, however, is not biologically meaningful; biologists are typically more interested in statements such as: 1) the number of viral particles in a given amount of blood, or 2) the fold change of p53 mRNA in an "equivalent amount" of cancerous vs. normal tissue. The analysis methods that address these two scenarios are commonly known as absolute quantification and relative quantification, respectively.

**Absolute quantification** is achieved by comparing the $C_T$ values of the test samples to a standard curve. The result of the analysis is quantity of nucleic acid (copy number, µg) per given amount of sample (per cell, per µg of total RNA). In **relative quantification**, the analysis result is a ratio: the relative amount (fold difference) of a target nucleic acid for equivalent amounts of test and control sample A vs. B. Both cases need to address the question of what the "amount of sample" is, and in relative quantification, to ensure that equivalent amounts of samples are compared.

For both absolute and relative quantification methods, quantities obtained from a qPCR experiment must be normalized in such a way that the data become biologically meaningful. This is done through the use of **normalizers**. In absolute quantification, normalizers are used to adjust or standardize the obtained target quantity to the unit amount of sample. In relative quantification, normalizers are used to ensure that the target quantities from equivalent amounts of samples are compared. Although the normalizer for either quantification method can be the number of cells used for template preparation, µg of nucleic acid used as PCR template, or the expression level of a reference gene, the first two are more commonly used for absolute quantification, whereas the third is typically used for relative quantification. A reference gene is one whose expression level is constant across all test samples and whose expression is not affected by the experimental treatment under study. The use of a reference gene is advantageous in cases where the precise quantification of input RNA amount is not possible, such as in cases where only a small amount of starting template is available.

Many different methods are available for performing both absolute and relative quantification. In the next few sections, we provide examples and guidelines on when and how to use absolute vs. relative quantification methods, and further explore the concept of normalizer as applied to each of the two quantification strategies.

# 4.1 Absolute Quantification

## 4.1.1 When Should Absolute Quantification Be Used?

Let's start by examining a sample case that calls for absolute quantification. A doctor is interested in determining the number of viral particles per ml of blood from a patient. To do so, the doctor extracts DNA from 10 ml of blood and determines the number of viral particles by running a real-time PCR assay specific to the viral DNA. By comparing the $C_T$ value obtained for the test sample to a standard curve of known amounts of viral particles, the doctor can interpolate that there are 10,000 viral particles in the assayed DNA sample. Based on this information, she is then able to determine that there are 1,000 viral particles/ml of blood.

In the above example, absolute quantification was chosen because the doctor was interested in the quantity of nucleic acid (number of viral particles) per given amount of sample (ml of blood). Thus, the normalizer is "ml of blood" in this example. The end result is a quantitative description of a single sample, which does not depend on the properties of any other sample. In other words, use the absolute quantification method when you are interested in finding out an intrinsic property of a given sample.

## 4.1.2 Absolute Quantification Using a Standard Curve

In absolute quantification, the quantity (e.g., copy number or unit mass) of the unknown sample is interpolated from a range of standards of known quantity. To construct a standard curve, a template with known concentration is required. Dilution of this template is then performed and these dilutions serve as the standards. The unknown test samples are assayed with the standards in the same experimental run. The standard curve constructed from the diluted standard template can then be used to determine the target quantity in the unknown sample by interpolation, similarly to using molecular size standards to determine the molecular size of an unknown DNA band on an agarose gel.

An example of using a standard curve to determine the copy number of a particular target nucleic acid is shown in Figure 4.1. In this example, we were interested in determining the copy number of the β-actin gene in two samples, A and B. A 10-fold dilution series was created from a sample with a known concentration of β-actin, resulting in a set of standards containing $10^2$–$10^7$ copies of template. The standards and the samples were then assayed in the same run.

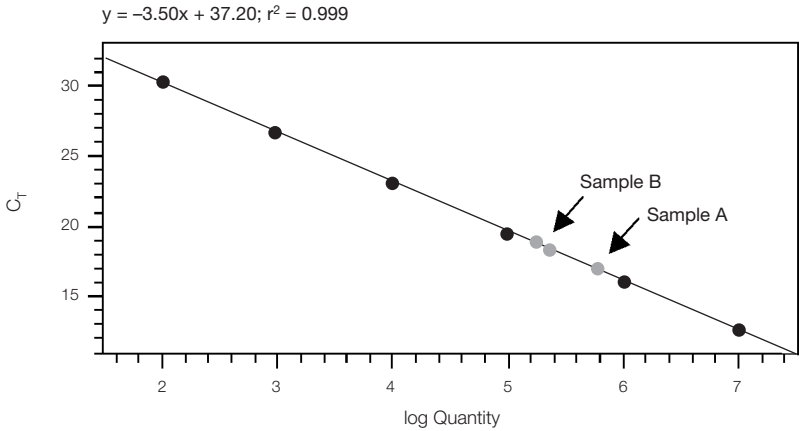



**Fig. 4.1. An example of absolute quantification using a standard curve.** To determine the copy number of β-actin in unknown samples A and B, 10-fold dilutions of a known concentration of template ($10^2$–$10^7$ copies β-actin) were assayed with the two unknown samples in the same run using a SYBR Green I assay specific to β-actin. The regression line from the dilution curve was used to determine the copy number of the unknown samples. All standard dilutions and unknown samples were assayed in triplicate on the Chromo4 real-time PCR detection system.

A standard curve was constructed, with the logarithm of the initial copy number of the standards plotted along the x-axis and their respective $C_T$ values plotted along the y-axis (Figure 4.1). The equation for the linear regression line [y = mx + b, or $C_T$ = m(log quantity) + b] is shown on the top of the graph.

Based on the equation for the linear regression, we can derive the following equation to determine the quantity of an unknown sample:

$$N_n = 10^{\left(\frac{n-b}{m}\right)}, \text{ where } n = C_T$$

$$\text{Quantity} = 10^{\left(\frac{C_T-b}{m}\right)}$$

**Table 4.1. Determination of the absolute copy number of β-actin in unknown samples A and B using the absolute quantification method.** The equation of the linear regression line shown in Figure 4.1 was used to calculate the copy number of the unknown samples.

| Sample | Replicate | $C_T$ | Copies |
|---|---|---|---|
| A | 1 | 18.61 | 204,577 |
| A | 2 | 18.41 | 234,115 |
| A | 3 | 18.87 | 172,300 |
| **Average** | | | **203,664 ± 30,917** |
| B | 1 | 17.06 | 569,789 |
| B | 2 | 17.07 | 563,823 |
| B | 3 | 17.00 | 591,173 |
| **Average** | | | **574,928 ± 14,381** |

The copy numbers of the individual replicate assays for samples A and B were determined using their $C_T$ values in the above equation. Typically, when replicates are sampled, a mean and standard deviation of the individual samples is reported, as shown in the last column of Table 4.1. It is important to note that the standard curve may only be used to interpolate, not extrapolate, the quantity of the unknown sample. This is because the assay may not be linear outside the range covered by the standards tested.

Common real-time PCR applications that employ absolute quantification include chromosome or gene copy number determination and viral load determination. This method of quantification is conceptually simple and the mathematics of the analysis is easy to perform. The method does, however, require a reliable source of template of known concentration, and standards must be amplified in parallel with the samples every time the experiment is performed.

# 4.2 Relative Quantification

## 4.2.1 When Should Relative Quantification Be Used?

Now let's examine a sample case that calls for relative quantification. A researcher wants to know whether the expression level of p53 differs between cancerous and normal ovarian cells, and if so, by how much. He wants to find out the relative amount, sometimes called the "fold difference", of p53 mRNA in equivalent amounts of the two tissues. The researcher can choose one of the following experimental designs depending on the type of normalizer he wishes to employ:

(1) The researcher extracts RNA from 1,000 cancerous and 1,000 normal ovarian cells, and determines the amount of p53 mRNA in the samples by running a reverse transcription (RT) qPCR assay specific for p53 mRNA. The researcher then finds out the relative fold difference of p53 mRNA by taking the ratio of the number of p53 mRNA molecules in RNA samples from the equivalent number of cancerous and normal ovarian cells.

(2) If the researcher had previously determined that the level of GAPDH mRNA expression is identical in cancerous and normal cells, the researcher can extract RNA from an approximately equivalent number of cancerous and normal ovarian cells without determining the exact number of cells. The level of p53 mRNA and GAPDH mRNA in the samples were then determined by a RT-qPCR assay. The relative expression of p53 in cancerous vs. normal cells was determined by taking the ratio of the GAPDH-normalized p53 expression in the two different cell types.

In the above example, relative quantification was chosen because the researcher was interested in the relative amount of a target nucleic acid in equivalent amounts of samples A and B. The difference between the two analysis methods described above is the type of normalizer used. In the first scenario, the normalizer is the number of cells. In the second scenario, the normalizer is the GAPDH mRNA expression level, which was previously determined to be constant between the two cell types. The mathematical manipulations for the two scenarios are presented in Sections 4.2.2 and 4.2.3, respectively. In both cases, the end result is a quantitative description of some property of one sample relative to the same property of another sample. In other words, use relative quantification to compare a specific property between multiple samples.

### 4.2.2 Relative Quantification Normalized Against Unit Mass

The advantages of using a unit mass (such as cell number or µg of nucleic acid) rather than a reference gene as the normalizer are that the experimental design is conceptually simple and the mathematical treatment is straightforward. The method requires accurate quantification of the starting material, regardless of whether cell number or µg of nucleic acid is used as the normalizer.

When comparing multiple samples using relative quantification, one sample is usually chosen as the calibrator (sometimes known as the control sample), and the expression of the target gene in all other samples is expressed as an increase or decrease relative to the calibrator. Customarily, the untreated or baseline sample is chosen as the calibrator. In the case of the p53 example, we may choose the normal ovarian cells as the calibrator and the cancerous ovarian cells as the test sample.

To determine the relative expression of p53 in cancerous vs. normal ovarian cells, total RNA was prepared from an equal number of normal ovarian cells and cancerous ovarian cells. The $C_T$ values for the test sample (cancerous cells) and the calibrator sample (normal cells) were then used to calculate the ratio between the two by the following equation:

$$\text{Ratio}_{(\text{test/calibrator})} = E^{C_T(\text{calibrator}) - C_T(\text{test})}$$

E is the efficiency of the reaction and can be determined as described in Section 1.1.3. If we assume that the assay has perfect amplification efficiency (that is, the template is doubled in each amplification cycle), then the equation above becomes:

$$\text{Ratio}_{(\text{test/calibrator})} = 2^{C_T(\text{calibrator}) - C_T(\text{test})}$$

or

$$\text{Ratio}_{(\text{test/calibrator})} = 2^{\Delta C_T}$$

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

This method for calculating relative expression is often referred to as the $\Delta C_T$ method. A hypothetical example is given below to demonstrate how the $\Delta C_T$ method can be used to determine relative expression of a target gene (p53) in cancerous and normal ovarian cells.

*Example*: cDNA representing 50 ng of total RNA isolated from both normal and tumor ovarian cells was assayed for p53 message. The $C_T$ values for each sample are shown below:

| Sample | $C_T$ for p53 (target) |
|---|---|
| Normal (calibrator) | 15.0 |
| Tumor (test) | 12.0 |

Assuming 100% amplification efficiency (E = 2), and choosing the normal cells as the calibrator, the relative expression of p53 expression in cancerous vs. normal ovarian cells is calculated as follows:

$$\text{Ratio}_{(\text{test/calibrator})} = 2^{C_T(\text{calibrator}) - C_T(\text{test})}$$
$$= 2^{(15 - 12)}$$
$$= 8$$

Therefore, the expression of p53 is 8-fold higher in tumor cells than in normal ovarian cells.

### 4.2.3 Relative Quantification Normalized to a Reference Gene

The advantage of using a reference gene (such as GAPDH, β-actin, etc.) rather than unit mass as a normalizer is that this method circumvents the need for accurate quantification and loading of the starting material. This is especially convenient when performing relative gene expression experiments where starting material is frequently limited. The drawback is that this method requires the availability of a known reference gene or genes with constant expression in all samples tested and whose expression is not changed by the treatment under study. The identification of such a reference gene is not trivial, and recently it has been proposed that in most cases, the use of multiple reference genes may be necessary for accurate quantification (see Vandesompele et al., Genome Biology 3, research 0034.1–0034.11, 2002, for detailed discussion).

As discussed in the previous section, when comparing multiple samples using relative quantification, one of the samples is usually chosen as the calibrator, and the expression of the target gene in all other samples is expressed as an increase or decrease relative to the calibrator. Customarily, the untreated or baseline sample is chosen as the calibrator. In the case of the p53 example, we may choose the normal ovarian cells as the calibrator and the cancerous ovarian cells as the test sample. To determine the relative expression of a target gene in the test sample and calibrator sample using reference gene(s) as the normalizer, the expression levels of both the target and the reference genes need to be determined using RT-qPCR. In short, you need to determine the $C_T$ values, as shown in Table 4.2.

**Table 4.2. $C_T$ values required for relative quantification with reference gene as the normalizer.**

| | Test | Calibrator (cal) |
|---|---|---|
| Target gene | $C_{T(target, test)}$ | $C_{T(target, cal)}$ |
| Reference gene | $C_{T(ref, test)}$ | $C_{T(ref, cal)}$ |

After the $C_T$ values are measured, different methods can be used to determine the expression level of the target gene in the test sample relative to the calibrator sample. In the following sections, we present three methods for relative quantification using a reference gene: 1) the Livak method, also known as the $2^{-\Delta\Delta C_T}$ method, 2) the $\Delta C_T$ method using a reference gene, and 3) the Pfaffl method. Each method has advantages and disadvantages, as well as assumptions that must be satisfied for the results of the analysis to be valid.

### 4.2.3.1 The $2^{-\Delta\Delta C_T}$ (Livak) Method

The $2^{-\Delta\Delta C_T}$ method for relative gene expression analysis is widely used and easy to perform. This method assumes that both target and reference genes are amplified with efficiencies near 100% and within 5% of each other. Before using the $2^{-\Delta\Delta C_T}$ method, it is essential to verify the assumptions by determining the amplification efficiencies of the target and the reference genes. The amplification efficiency of the target and the reference gene can be determined by the method outlined in Section 1.1.3.

Once you have established that the target and reference genes have similar and nearly 100% amplification efficiencies, you can determine the relative difference in expression level of your target gene in different samples using the steps below:

First, normalize the $C_T$ of the target gene to that of the reference (ref) gene, for both the test sample and the calibrator sample:

$$\Delta C_{T(test)} = C_{T(target,\ test)} - C_{T(ref,\ test)}$$

$$\Delta C_{T(calibrator)} = C_{T(target,\ calibrator)} - C_{T(ref,\ calibrator)}$$

Second, normalize the $\Delta C_T$ of the test sample to the $\Delta C_T$ of the calibrator:

$$\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(calibrator)}$$

Finally, calculate the expression ratio:

$$2^{-\Delta\Delta C_T} = \text{Normalized expression ratio}$$

The result obtained is the fold increase (or decrease) of the target gene in the test sample relative to the calibrator sample and is normalized to the expression of a reference gene. Normalizing the expression of the target gene to that of the reference gene compensates for any difference in the amount of sample tissue.

If the target and the reference genes do not have similar amplification efficiencies you can either optimize or redesign the assays, or you can use the Pfaffl method described in Section 4.2.3.3. If, on the other hand, the target and the reference genes have identical amplification efficiency, but the efficiency is not equal to 2, a modified form of the $2^{-\Delta\Delta C_T}$ method may be used by replacing the 2 in the equation by the actual amplification efficiency. For example, if the amplification efficiency of both the target and the reference gene is 1.95, the formula $1.95^{-\Delta\Delta C_T}$ should be used.

The following hypothetical example demonstrates how the $2^{-\Delta\Delta C_T}$ method is used to determine relative expression of a target gene (p53) in cancerous and normal ovarian cells.

*Example:* cDNAs representing 50 ng of total RNA isolated from both normal and tumor ovarian cells were assayed for p53 (target gene) and GAPDH (reference gene) message. GAPDH can serve as a reference gene for this study because previous studies showed that GAPDH expression does not vary between normal and tumor cells. The $C_T$ values for each sample are shown below:

| Sample | $C_T$ p53 (target) | $C_T$ GAPDH (reference) |
|---|---|---|
| Normal (calibrator) | 15.0 | 16.5 |
| Tumor (test) | 12.0 | 15.9 |

To calculate relative expression using the steps described above, the $C_T$ of the target gene is normalized to the $C_T$ of the reference gene for both the test sample and the calibrator sample:

$$\Delta C_{T(normal)} = 15.0 - 16.5 = -1.5$$

$$\Delta C_{T(tumor)} = 12.0 - 15.9 = -3.9$$

Second, the $\Delta C_T$ of the test sample is normalized to the $\Delta C_T$ of the calibrator:

$$\Delta\Delta C_T = \Delta C_{T(tumor)} - \Delta C_{T(normal)}$$
$$= -3.9 - (-1.5) = -2.4$$

Finally, the expression ratio is calculated:

$$2^{-\Delta\Delta C_T} = 2^{-(-2.4)} = 5.3$$

Tumor cells are expressing p53 at a 5.3-fold higher level than normal cells.

### 4.2.3.2 The $\Delta C_T$ Method Using a Reference Gene

The $\Delta C_T$ method using a reference gene is a variation of the Livak method that is simpler to perform and gives essentially the same results. In contrast to the $\Delta C_T$ values obtained in Section 4.2.2, this method uses the difference between reference and target $C_T$ values for each sample. Despite the simplicity of the approach, it is indeed normalized expression. The expression level of the reference gene is taken into account. The key difference in the results is that the expression value of the calibrator sample is not 1.0. If the resulting expression values obtained in this method are divided by the expression value of a chosen calibrator, the results of this calculation are exactly the same as those obtained with the $2^{-\Delta\Delta C_T}$ method:

$$\text{Ratio (reference/target)} = 2^{C_T(\text{reference}) - C_T(\text{target})}$$

The mathematical assumptions for this approach are the same as those for the $2^{-\Delta\Delta C_T}$ method.

*Example:* cDNA representing 50 ng of total RNA isolated from both normal and tumor ovarian cells was assayed in triplicate for p53 and GAPDH message. Previous studies have concluded that GAPDH is a good reference gene for this study. The results are shown below:

| Sample | $C_T$ p53 (target) | $C_T$ GAPDH (reference) |
|---|---|---|
| Normal (calibrator) | 15.0 | 16.5 |
| Tumor (test) | 12.0 | 15.9 |

To calculate relative expression, simply normalize p53 expression for each sample:

$2^{(C_T(GAPDH) - C_T(p53))}$ = Expression

For normal cells, this yields $2^{(16.5 - 15)} = 2.8$

For tumor cells, this yields $2^{(15.9 - 12)} = 14.9$

Although at first glance, these results appear quite different from those obtained using the $2^{-\Delta\Delta C_T}$ method, a quick evaluation of the ratio between the two samples will reveal that these give precisely the same results:

Normal expression = Normal/Normal = 2.8/2.8 = 1

Tumor expression = Tumor/Normal = 14.9/2.8 = 5.3

### 4.2.3.3 The Pfaffl Method

The $2^{-\Delta\Delta C_T}$ method for calculating relative gene expression is only valid when the amplification efficiencies of the target and reference genes are similar. If the amplification efficiencies of the two amplicons are not the same, an alternative formula must be used to determine the relative expression of the target gene in different samples. To determine the expression ratio between the sample and calibrator, use the following formula:

$$\text{Ratio} = \frac{(E_{target})^{\Delta C_T, \text{ target (calibrator – test)}}}{(E_{ref})^{\Delta C_T, \text{ ref (calibrator – test)}}}$$

In the above equation, $E_{target}$ and $E_{ref}$ are the amplification efficiencies of the target and reference genes, respectively. $\Delta C_T$, target (calibrator – test) = $C_T$ of the target gene in the calibrator minus the $C_T$ of the target gene in the test sample, and $\Delta C_T$, ref (calibrator – test) is the $C_T$ of the reference gene in the calibrator minus the $C_T$ of the reference gene in the test sample.

The above equation assumes that each gene (target and reference) has the same amplification efficiency in test samples and calibrator samples, but it is not necessary that the target and reference genes have the same amplification efficiency as each other.

The $2^{-\Delta\Delta C_T}$ method and the Pfaffl method are very closely related; in fact, the $2^{-\Delta\Delta C_T}$ method is simply a special case of the Pfaffl method where $E_{target} = E_{ref} = 2$. If we substitute $E_{target}$ and $E_{ref}$ with 2, then the Pfaffl method simplifies as follows:

$$\text{Ratio} = \frac{2^{\Delta C_T,\ \text{target (calibrator – test)}}}{2^{\Delta C_T,\ \text{ref (calibrator – test)}}}$$

$$= 2^{-[(C_T,\ \text{target (test)} - C_T,\ \text{target (calibrator)}] - [(C_T,\ \text{ref (test)} - C_T,\ \text{ref (calibrator)}]}$$

$$= 2^{-\Delta\Delta C_T}$$

# Gene Expression Analysis

# 5. Gene Expression Analysis

The most common application of real-time PCR is the study of gene expression. Measuring gene expression is of fundamental importance in many areas of contemporary biomedical research, ranging from basic science to practical applications in industry and medicine. In most gene expression studies, researchers are interested in determining the relative gene expression levels in test vs. control samples. For example, a researcher might be interested in the expression of a particular gene in cancerous vs. normal tissue. In this section, general guidelines and a sample gene expression experiment are presented to demonstrate how to perform gene expression analysis using real-time PCR.

## 5.1 Experimental Design

A sample gene expression analysis using a multiplex TaqMan assay is presented in the following sections. In this example, we're interested in the relative expression of three genes in the polyamine biosynthesis pathway, ornithine decarboxylase (ODC), ODC antizyme (OAZ), and antizyme inhibitor (AZI), in two different samples, sample A and sample B. Due to possible pipetting errors and initial quantification errors of the input RNA, the amount of starting cDNA in the different real-time reactions may be different. In this example, the expression of a reference gene, β-actin, was chosen as the normalizer to control for any difference in cDNA input amount. The following steps were performed to determine the relative expression level of ODC, OAZ, and AZI in the two different samples:

1. RNA was isolated from sample A and sample B.
2. RNA was reverse transcribed into cDNA.
3. The amount of the target genes (ODC, OAZ, and AZI) and the reference gene (β-actin) was determined in each of the cDNA samples using a multiplex qPCR assay.
4. Data were analyzed and the relative expression of each of the target genes in the two samples was calculated.

This workflow, which is typical of RT-qPCR experiments, is summarized in Figure 5.1. In the following sections, we will go over practical considerations of performing each step shown in the gene expression analysis workflow.

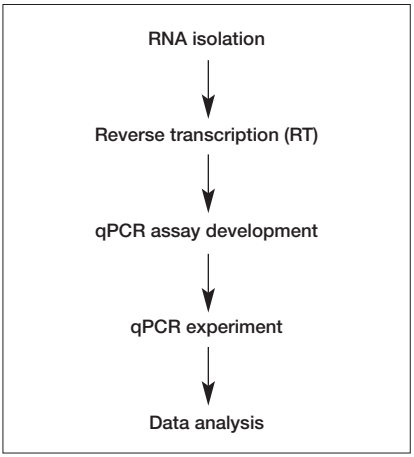



**Fig. 5.1. Gene expression analysis workflow.**

# 5.2 RNA Isolation

## 5.2.1 Sample Collection

For quantifying gene expression, sample material should be as homogeneous as possible. If your tissue sample consists of many different cell types, pinpointing the expression pattern of your target gene may be difficult. If you have a heterogeneous sample, use one of the many methods that are available for separating and isolating specific cell types. These methods include tissue dissection, needle biopsies, and laser capture microdissection. The collected cells can then be used to obtain the RNA samples.

## 5.2.2 RNA Extraction

Either total or poly(A+) RNA can be used for most real-time RT-qPCR applications. One critical consideration in working with RNA is to avoid RNases in your solutions, consumables, and labware. Ready-to-use solutions that are RNase-free can be purchased. Alternatively, treat solutions with diethyl pyrocarbonate (DEPC), and then autoclave them. RNases on labware can also be inactivated by DEPC treatment, or by baking at 250°C for 3 hr. RNase-free consumables are available for purchase from many commercial sources.

Prepared RNA samples may need DNase treatment to prevent potential amplification of any contaminating genomic DNA, which could lead to overestimation of the copy number of an mRNA. When starting material is limited, however, DNase treatment may be inadvisable, because the additional manipulation could result in loss of RNA. The amplification of potential contaminating genomic DNA can be precluded by designing transcript-specific primers, for example, primers that span or amplify across splice junctions.

## 5.2.3 Analyzing Nucleic Acid Quantity and Quality

Accurate nucleic acid quantification is essential for gene expression analysis, especially if total RNA amounts are used to normalize target gene expression. RNA concentrations and purity are commonly determined by measuring the ratio of the UV absorbance at 260 nm and 280 nm. The overall sensitivity of this method is low, especially for relatively dilute samples, and it doesn't indicate the quality of the RNA. To determine the quality and quantity of your sample, we recommend using the Experion™ automated electrophoresis system (see Product Guide). Examples of a high-quality total RNA preparation and a poor-quality total RNA preparation are shown in Figure 5.2.

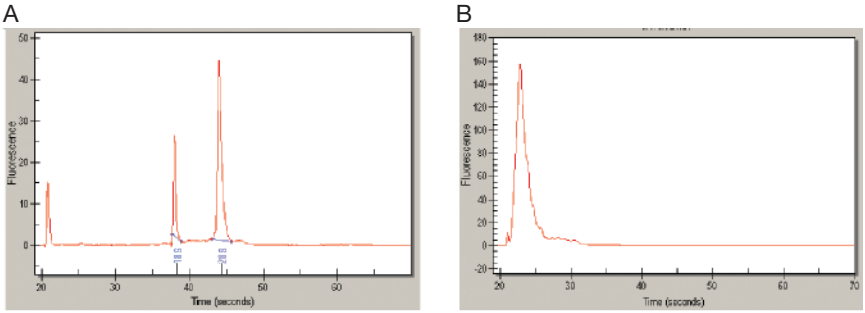



**Fig. 5.2. RNA quality assessed by an Experion electrophoresis system. A**, An example of a high-quality total RNA preparation showing distinct peaks for the 18S and 28S rRNA; **B**, an example of a poor-quality total RNA preparation where only small, degraded fragments are observed.

# 5.3 cDNA Template Preparation (Reverse Transcription)

Two methods are available for quantification of gene expression by RT-qPCR: "two-step" RT-qPCR or "one-step" RT-qPCR. In both cases, RNA is reverse transcribed into cDNA, and the cDNA is then used as the template for quantitative PCR amplification. "One-step" and "two-step" refers to whether the reverse transcription and real-time PCR amplification are done in the same or separate tubes. In the two-step method, RNA is first transcribed into cDNA in a reaction using reverse transcriptase. An aliquot of the resulting cDNA can then be used as a template source for multiple qPCR reactions. In the one-step method, reverse transcription and qPCR are performed in the same tube.

In two-step RT-qPCR, the RT step can be primed with oligo(dT) primers, random primers, a mixture of the two, or gene-specific primers. One-step RT-PCR must be performed using gene-specific primers, and can be achieved either by using *Thermus thermophilus* (*Tth*) polymerase, a DNA polymerase with inherent RT activity, or by a two-enzyme system combining a reverse transcriptase with a thermostable DNA polymerase. Since *Tth* DNA polymerase is derived from a thermophilic bacterium, higher temperatures (>60ºC) can be used for the RT step, which can minimize secondary structure in high-GC-content mRNAs.

Accurate analysis of gene expression requires good reproducibility of reverse transcription reactions. The robustness of a reverse transcription is determined by the sensitivity, dynamic range, and specificity of the reverse transcriptase used. Reverse transcriptases from Moloney murine leukemia virus (MMLV) and avian myeloblastoma virus (AMV) are the most commonly used enzymes. When long or full-length cDNA transcripts are needed, MMLV reverse transcriptase and its derivatives are better choices than AMV reverse transcriptase due to their lower RNase H activity. It is has been observed, however, that RT reactions performed with RNase $H^-$ reverse transcriptases sometimes require subsequent RNase H treatment to effectively amplify certain templates and those at low concentration in the PCR.

As noted above, the reverse transcription step in two-step RT-qPCR can be performed using oligo(dT) primers, random primers, a mix of these two, or gene-specific primers. The choice of primers may influence quantification of your target gene. Gene-specific primers give less background priming than oligo(dT) or random primers. If you choose oligo(dT) primers for reverse transcription, you may want to place PCR primers close to the 3' end of the transcript to avoid loss of sensitivity due to truncated messages; this is especially important for longer transcripts. Oligo(dT) priming should be avoided if you are working with transcripts or species that have short poly(A) tails or lack them altogether. For the 5'-nuclease assay, we found oligo(dT)–primed cDNA to perform very well; however, random-primed cDNA performed equally well or slightly better for most sequences, and much better for some sequences. For most PCR applications, we have found best performance using iScript™ and iScript™ Select cDNA synthesis kits, which employ an MMLV RT (see Product Guide).

## 5.4 qPCR Assay Development

Gene expression analyses may be performed as singleplex or multiplex assays, using many different types of chemistries. Your experimental design will depend on both practical and scientific considerations. Some factors to consider when deciding between singleplex and multiplex assays are discussed in Section 2.2.1. For singleplex assays, any chemistry listed in Section 2.2.2 can be used. For multiplex assays, one must use a fluorescent primer- or probe-based chemistry, such as those listed in Section 2.2.2.2; dsDNA binding dyes are not compatible with multiplex assays.

Details on how to design and optimize singleplex and multiplex TaqMan assays are given in Section 2.4 and Section 3; refer to those sections for details on assay development and optimization. Note that for gene expression experiments, primers should be designed to span splice junctions to avoid amplifying genomic DNA.

# 5.5 Experimentation

To determine the expression of the three target genes, ODC, OAZ, and AZI, and the reference gene, β-actin, in samples A and B, these four genes were assessed in a multiplex two-step RT-qPCR assay.

## 5.5.1 Reaction Components for Multiplex Assay

The multiplex assay contained the following components in a 50 µl reaction:

1/10 of a reverse transcription reaction that used 100 ng of total RNA
300 nM each primer
200 nM each probe
200 µM each dNTP
5 mM $MgCl_2$
3.75 U iTaq™ DNA polymerase

## 5.5.2 Cycling Protocol

The cycling program used is shown below:

| | | |
|---|---|---|
| Cycle 1 | 1 repeat | 95°C for 3 min |
| Cycle 2 | 40 repeats | |
| Step 1 | | 95°C for 10 sec |
| Step 2 | | 59°C for 60 sec (data collection) |

# 5.6 Gene Expression Data Analysis

The relative expression of ODC, OAZ, and AZI, with β-actin as the reference gene, was determined using the $2^{-\Delta\Delta C_T}$ (Livak) method described in Section 4.2.3.1. Results are shown in Table 5.1. Amplification efficiencies for all four genes were previously shown to be equal and >99.5%. Because all four genes were assayed in a single reaction, the β-actin gene and each of the three target genes, ODC, AZI, and OAZ, could be considered paired data points; therefore, $\Delta C_T$ between the reference and target genes was first calculated for each replicate, and then averaged across the three replicates. The average $\Delta C_T$ for each target gene from the two tissue samples was then used to calculate $\Delta\Delta C_T$, and this, in turn, was used to calculate the fold difference of the three target genes between the two samples. The respective levels of ODC, AZI, and OAZ were found to be 7.0–8.0-fold, 2.8–3.7-fold, and 3.0–3.5-fold higher in sample B relative to sample A.

**Table 5.1. Relative quantification of ODC, OAZ, and AZI in sample B relative to sample A.**

| | | $C_T$ Values | | | |
|---|---|---|---|---|---|
| **Sample** | | **β-Actin** | **ODC** | **AZI** | **OAZ** |
| A | Replicate 1 | 20.3 | 27.9 | 25.8 | 24.0 |
| | Replicate 2 | 20.2 | 28.0 | 25.9 | 24.2 |
| | Replicate 3 | 20.3 | 27.9 | 26.0 | 24.0 |
| **Average $\Delta C_T$** | | | **7.6 ± 0.1** | **5.6 ± 0.1** | **3.8 ± 0.1** |
| B | Replicate 1 | 20.8 | 25.5 | 24.9 | 22.9 |
| | Replicate 2 | 20.8 | 25.4 | 24.7 | 23.0 |
| | Replicate 3 | 20.9 | 25.6 | 24.6 | 22.9 |
| **Average $\Delta C_T$** | | | **4.7 ± 0.1** | **3.9 ± 0.2** | **2.1 ± 0.1** |
| Sample B $\Delta C_T$ – Sample A $\Delta C_T$ | | | –2.9 ± 0.1 | –1.7 ± 0.2 | –1.7 ± 0.1 |
| Fold difference ($2^{-\Delta\Delta C_T}$) | | | 7.0–8.0 | 2.8–3.7 | 3.0–3.5 |

# Genotyping/Allelic Discrimination

# 6. Genotyping/Allelic Discrimination

Single nucleotide polymorphisms (SNPs) are DNA sequence variations commonly found among individuals as a result of single base-pair substitutions. In general, an allelic sequence variation is considered a DNA polymorphism if more than one allele is found at a single locus with a frequency greater than 1% in the human population. DNA sequence variations play important roles in many processes, such as an individual's susceptibility to diseases and his or her response to drug therapies. SNPs are also useful in investigating the contributions of individual genes to diseases that have a complex multigene basis.

The identification of SNPs, and hence the study of human variation and diseases, has been facilitated by the development of molecular tools to determine DNA changes. SNP detection, genotyping, or allelic discrimination can be performed by methods such as allele-specific PCR, allele-specific primer extension, oligonucleotide ligation assay, and single-strand conformational polymorphism (SSCP) analysis. More recently, SNP genotyping methods based on real-time PCR have gained popularity in research and diagnostic laboratories. Real-time PCR technologies have many advantages over traditional methods of genotyping, including higher throughput, decreased risk of crossover contamination, and reduced labor.

In this chapter, we provide general guidelines for allelic discrimination using real-time PCR with TaqMan probes. A sample protocol for genotyping two risk factors of venous thrombosis in Caucasians is shown. The assay can be adapted easily to detect other SNPs.

# 6.1 Experimental Design

Genotyping and SNP detection assays using real-time PCR may be designed in many ways and with different probe-based chemistries (see Section 2.2.2.2), including TaqMan probes and molecular beacons. Below we discuss how TaqMan probes can discriminate between allelic variants, and we present special considerations for allelic discrimination with TaqMan probes, including primer and probe design, multiplexing, and validation.

How are TaqMan probes used to discriminate between allelic variants? The TaqMan probes used for allelic discrimination are differentially labeled fluorescent probes that are specific for each allele. One probe is specific for the wild-type (WT) allele and another probe is specific for an allelic variant. The probes are differentially labeled with a 5' fluorescent reporter dye and the appropriate 3' quencher (see Section 2.4.1 for reporter and quencher selection). As shown in Figure 6.1, the preferential hybridization of a perfectly matched probe versus a mismatched probe permits discrimination between the allelic variants. The matched TaqMan probe specifically hybridizes to the amplicon during the combined annealing/extension step, and the 5' exonuclease activity of the DNA polymerase cleaves off the fluorophore, separating it from the quencher to release a fluorescent signal for the matched allelic variant. The unbound probe of the mismatched allelic variant is not cleaved and remains quenched. The number of allelic variants detected will depend on your experimental design and the number of fluorescent dyes supported by your real-time instrument. The iCycler iQ®, Chromo4™, and iQ™5 systems are capable of simultaneously monitoring up to four different dyes and thus can discriminate up to four allelic variants using fluorescent allele-specific probes.

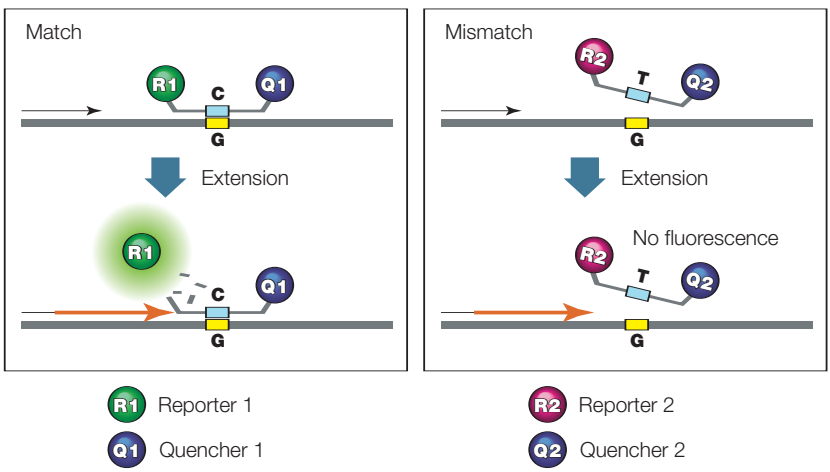



**Fig. 6.1. Mechanism of allelic discrimination using TaqMan assay.**

As mentioned above, assays for genotyping or SNP detection by real-time PCR may be designed in a variety of ways and will depend on your experimental system. For simple discrimination of two to four SNPs at a single nucleotide position using TaqMan probes, you would need to design a primer pair to amplify one amplicon and two to four allele-specific probes, each with a different fluorescent label. To detect SNPs at multiple nucleotide positions, single or multiple amplicons may be used. For example, a multiplex assay can be designed to coamplify two regions, and two allele-specific probe pairs would discriminate SNPs in each amplicon.

Below we describe a multicolor, multiplex real-time PCR assay using TaqMan probes for the simultaneous discrimination of allelic variants of the two most common genetic risk factors for venous thrombosis in Caucasians, factor V Leiden (FVL) and prothrombin (PT) G20210A mutations. This single-tube multiplex assay employs: 1) two primer pairs to simultaneously amplify sequences from the coagulation factor V gene (which has the FVL G1691A mutation) and from the coagulation factor II gene (which has the PT G20210A mutation), and 2) two pairs of allele-specific probes; each probe pair discriminates between the WT and the mutant allele for each target.

## 6.2 Primer and Probe Design Using TaqMan Probes

The guidelines presented in this section for primer and TaqMan probe design for allelic discrimination differ somewhat from the general guidelines in Section 2.4.1. To design primers for the allelic discrimination assays described here, we used Oligo 6 software (Molecular Biology Insights, Inc., Cascade, CO) and followed the general guidelines in Section 2.4.1 of this guide (avoid primer-dimers, hairpins, and self-complementary sequences), except that primers were designed with a $T_m$ of ~55°C. Primers were also designed to be compatible in a multiplex PCR reaction (that is, they were designed to have similar $T_m$ values and to avoid complementarity between all primers and probes; see Section 3.1). We designed two primer sets to coamplify a 97 bp sequence from the coagulation factor V gene and a 111 bp sequence from PT; each amplicon spanned the region to be genotyped (FVL and PT G20210A mutations). The primers used to amplify the coagulation factor V gene were: forward, 5'-TCT GAA AGG TTA CTT CAA GGA C-3' and reverse, 5'-ATC GCC TCT GGG CTA ATA-3'. The primers used to amplify PT were: forward, 5'-TTG TGT TTC TAA AAC TAT GGT TCC-3' and reverse, 5'-AGT AGT ATT ACT GGC TCT TCC T-3'.

For allelic discrimination, allele-specific TaqMan probes should be designed so that the probe for the matching allelic variant binds the target with high specificity, while the probe for the mismatched allelic variant should not bind. We have found that WT and mutant alleles can be effectively discriminated by designing probes that are short ($T_m$ ~55°C) and by placing the polymorphic nucleotide in the middle of the probe. Note that these probe design rules differ from those suggested by Livak (1999) and those discussed in Section 2.4.1 (probes with a $T_m$ of ~65–67°C, length ~23–26 bases; primers with a $T_m$ of 58–60°C). Although probes with high $T_m$ values work well for quantitative experiments, we recommend shorter TaqMan probes for allelic discrimination.

To detect the FVL and PT G20210A allelic variants, we designed probes complementary to sequences that span the mutations in the targets. Probes were designed to be relatively short (17–19 bases, $T_m$ = 55 ± 1.5°C), and the polymorphic nucleotide was placed in the center of the probes. Probes that discriminated between the coagulation factor V wild-type and the FVL mutant allele were 5'-[FAM]-TGG ACA GGC GAG GAA TAC-[Black Hole Quencher 1]-3' (for coagulation factor V wild type), and 5'-[TET]-TGG ACA GGC AAG GAA TAC A-[Black Hole Quencher 1]-3' (for FVL G20210A mutant). Probes that discriminated between the PT wild type and G20210A mutant allele were labeled with 5'-[TAMRA]-CTC TCA GCG AGC CTC AA-[Black Hole Quencher 2]-3' (for PT wild type), and 5'-[Texas Red]-ACT CTC AGC AAG CCT CAA-[Black Hole Quencher 2]- 3' (for PT G20210A mutant).

# 6.3 DNA Extraction and Sample Preparation

For SNP analysis and genotyping, human genomic DNA is typically prepared from whole blood, cell lines, or tissues (for detection of tumor mutations). We prepare our genomic DNA templates using either the AquaPure™ genomic DNA blood kit or the InstaGene™ matrix (see Product Guide). To facilitate the denaturation of intact, high molecular weight genomic DNA, you may need to prepare your genomic DNA template using the following steps: 1) digest with a restriction enzyme that does not cut within the region to be amplified, and/or 2) boil DNA stock for 10 min and place immediately on ice.

For the assay described here, whole blood samples from 52 individuals with FVL and PT G20210A genotypes previously determined by PCR-RFLP were used for the preparation of human genomic DNA using standard procedures. DNA concentrations and purity are generally measured by spectrophotometry (ratio of the UV absorbance at 260 nm and 280 nm) or a nucleic-acid dye-binding assay (e.g., Hoechst dye or PicoGreen for double-stranded DNA) and a fluorometer, such as the VersaFluor™ fluorometer (see Product Guide). Approximately 10 ng of DNA was used for each multicolor, multiplex reaction described here.

# 6.4 Reaction Components When Using TaqMan Probes

Although we added the individual components for the TaqMan allelic discrimination assay described here separately, preformulated PCR master mixes containing buffer, DNA polymerase, and dNTPs, such as iQ™ supermix, may also be used. Each reaction contains: 1) two primer sets (total of four primers) to coamplify sequences of the coagulation factor V gene and the PT gene, and 2) two pairs of differentially labeled allele-specific probes (total of four probes) to specifically detect WT and mutant alleles for each gene. Reaction components for the multiplex TaqMan allelic discrimination assay described here and their final concentrations are listed below:

10 ng DNA template
400 nM each primer
400 nM each probe
200 µM each dNTP
3 mM $MgCl_2$
20 mM Tris-HCl, pH 8.4
50 mM KCl
2.25 U iTaq™ DNA polymerase
Sterile, nuclease-free water to final volume of 25.0 µl

We recommend that you prepare a reaction master mix containing enough reagents to genotype the unknown DNA samples and controls, plus an excess to cover potential pipetting errors. For the most robust results, we also recommend genotyping at least three replicates of each of your controls (WT alleles, mutant alleles, and no-template control).

# 6.5 Optimization

To determine the annealing temperature that would yield the highest specific signals for each allele-specific probe, we took advantage of the gradient feature of the iCycler iQ® real-time PCR detection system. Wild-type and mutant control DNAs for each gene coagulation factor (V and PT), along with a no-template control, were tested in a temperature gradient optimization experiment. We examined eight temperatures, ranging from 55 to 70°C, in a single experiment. For this multiplex PCR reaction, we found that a combined primer/probe annealing and extension at 58°C for 45 sec gave the best discrimination between the allelic variants tested (data not shown).

# 6.6 Cycling Protocol Using TaqMan Probes

Coamplification of sequences that cover the FVL and the PT G20210A mutations was performed on the iCycler iQ system according to the protocol below. Fluorescence data generated by cleavage of the matching allele-specific probes were collected during the annealing/extension step at 58°C.

| | | |
|---|---|---|
| Cycle 1 | 1 repeat | 95°C for 3.5 min |
| Cycle 2 | 50 repeats | |
| Step 1 | | 95°C for 10 sec |
| Step 2 | | 58°C for 45 sec (data collection) |

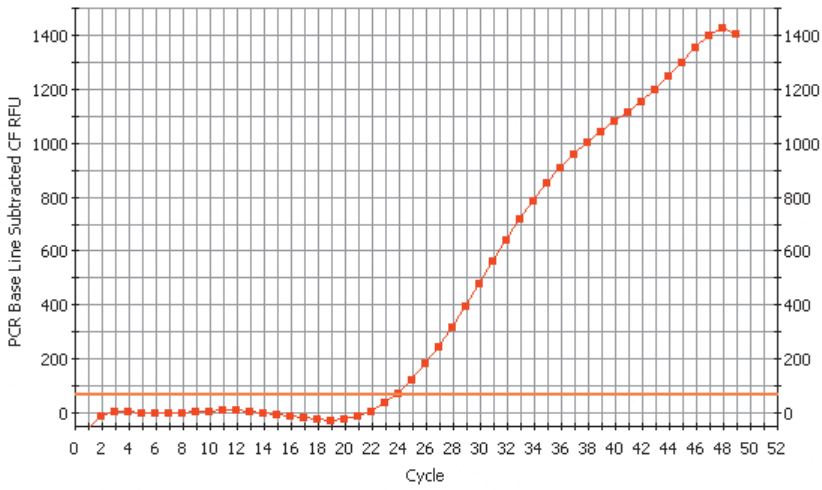
# 6.7 Allelic Discrimination Data Analysis

## 6.7.1 Validation of Allelic Discrimination Assay

We analyzed DNA from individuals with WT and mutant genotypes for FVL and PT G20210A mutations; the six possible outcomes are shown in Figures 6.2–6.7. A WT individual (Figure 6.2) shows a fluorescent signal for both WT probes but not for their mutant counterparts (FVL and PT G20210A). An individual who is homozygous for one of the two mutations (Figures 6.3 and 6.4) shows a fluorescent signal with the probe complementary to the mutant sequence but not with its WT counterpart, and another fluorescent signal with the other WT probe but not with its mutant counterpart. Individuals who are heterozygous for a single target generate a fluorescent signal with both probes for that target (Figures 6.5 and 6.6). Compound heterozygous individuals generate fluorescent signals with all four probes (two WT probes and two mutant counterpart probes; see Figure 6.7).

FVL WT probe/FAM




PT G20120A WT probe/TAMRA

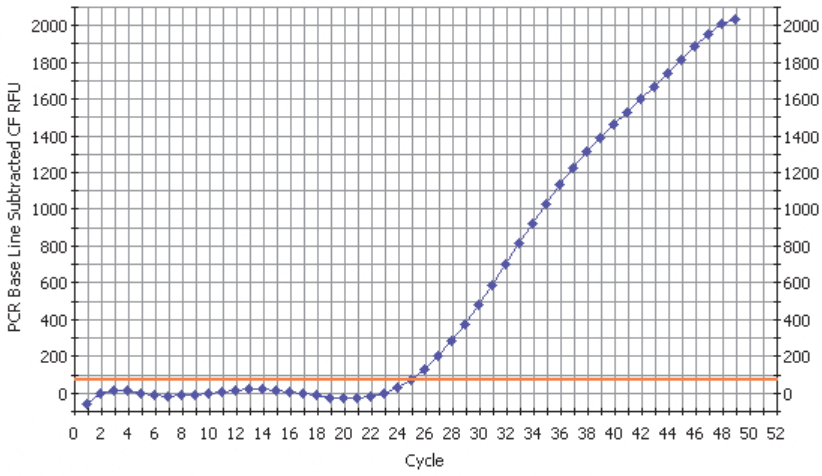
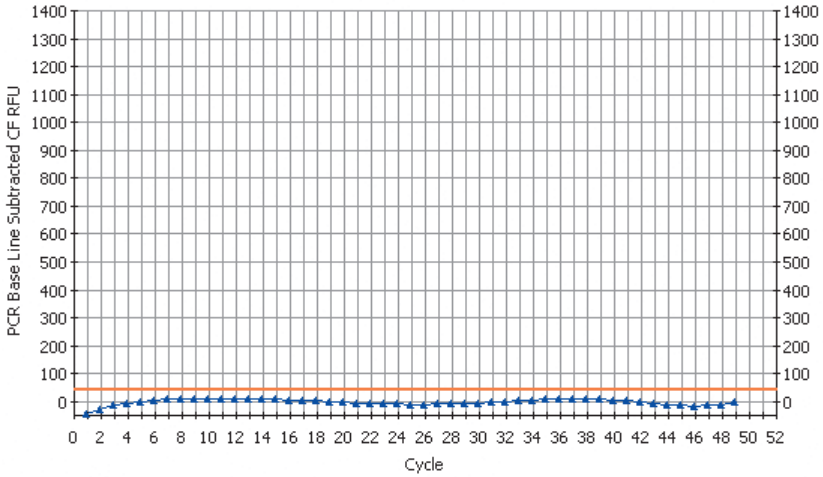


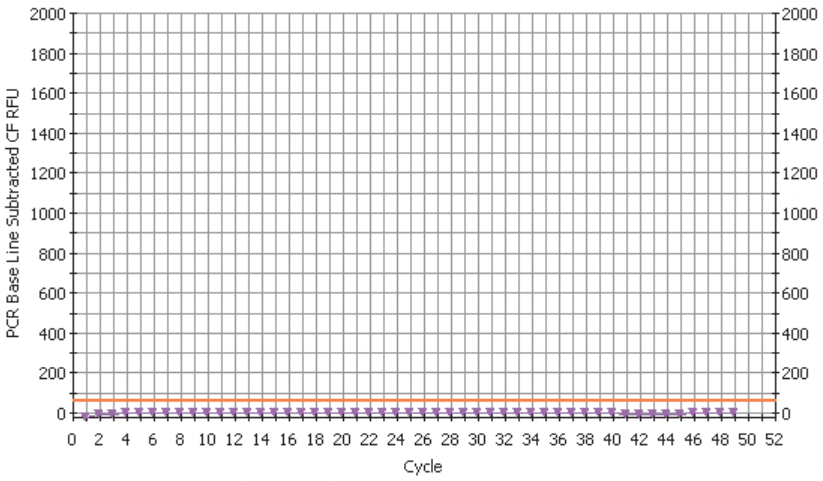

Fig. 6.2. WT genotype.

**FVL mutant probe/TET**




**PT G20210A mutant probe/Texas Red**



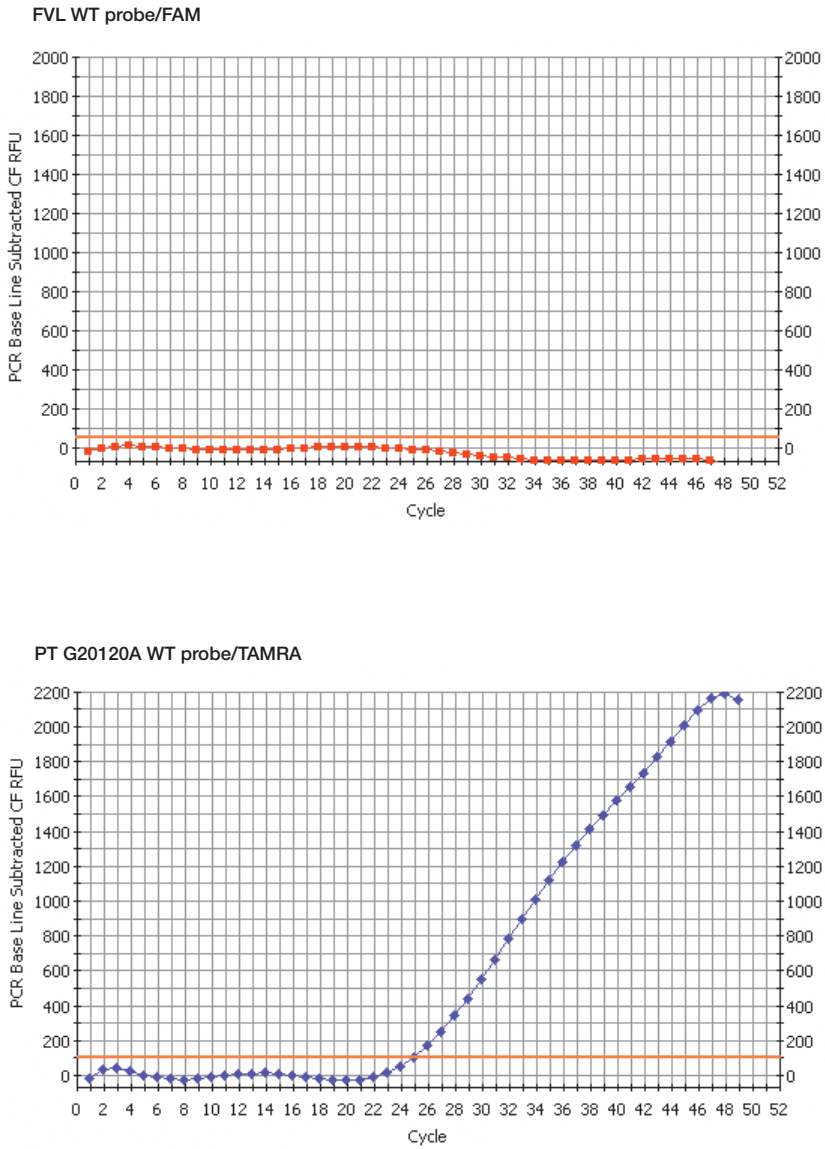
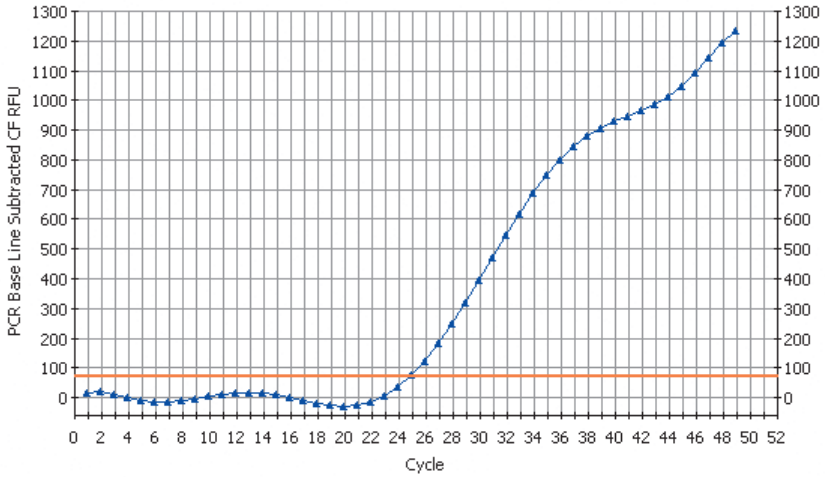


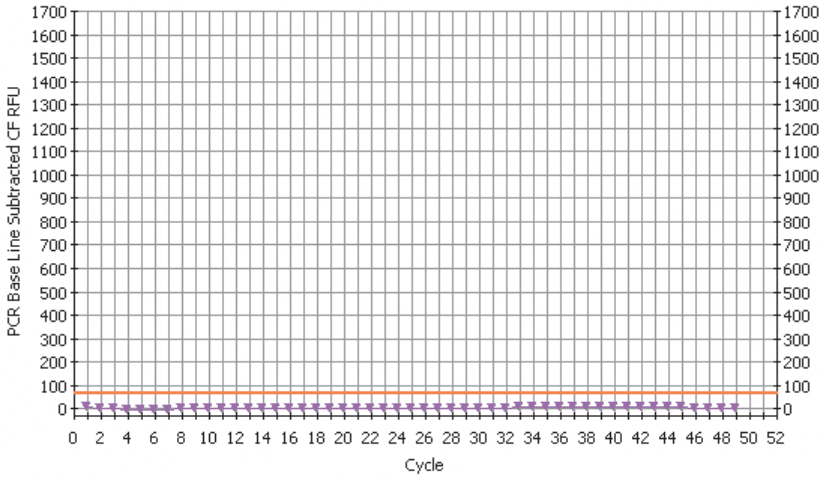

**Fig. 6.3. Homozygous mutant FVL.**

**FVL mutant probe/TET**




**PT G20210A mutant probe/Texas Red**



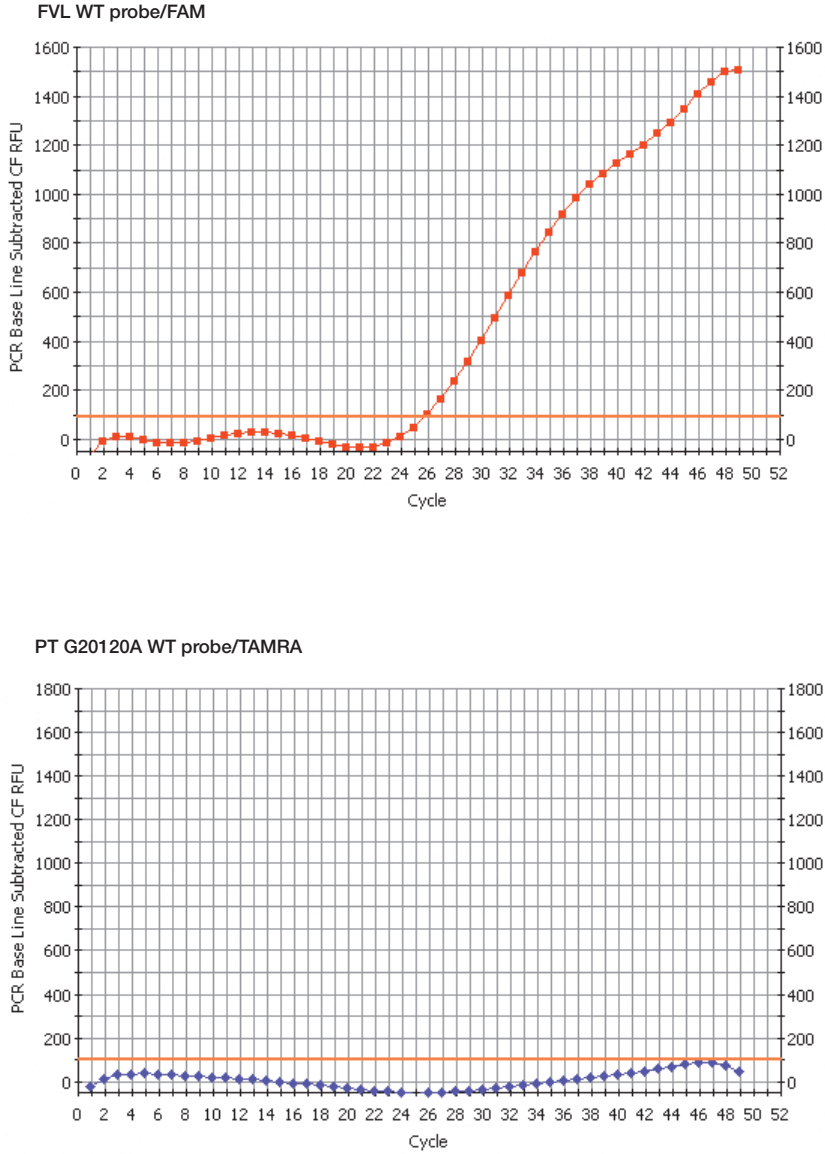
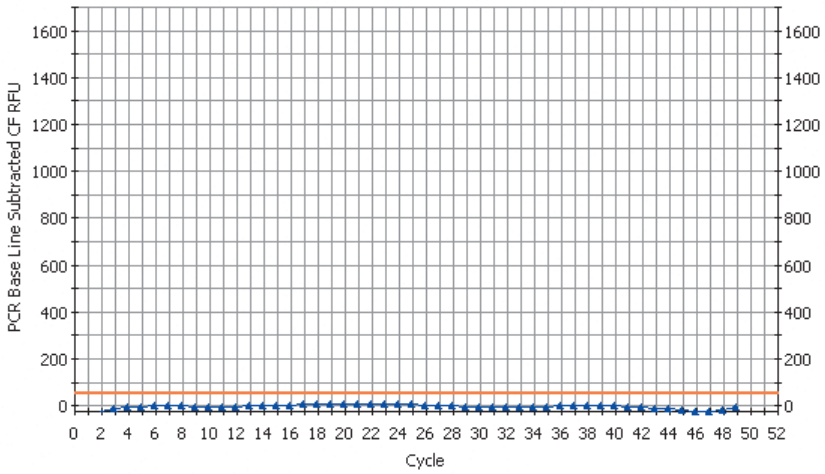


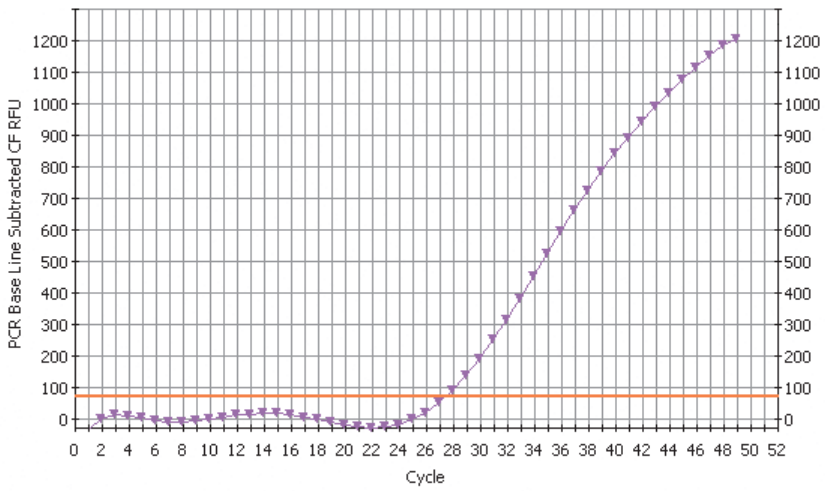

**Fig. 6.4. Homozygous mutant PT G20210A.**

FVL mutant probe/TET




PT G20210A mutant probe/Texas Red



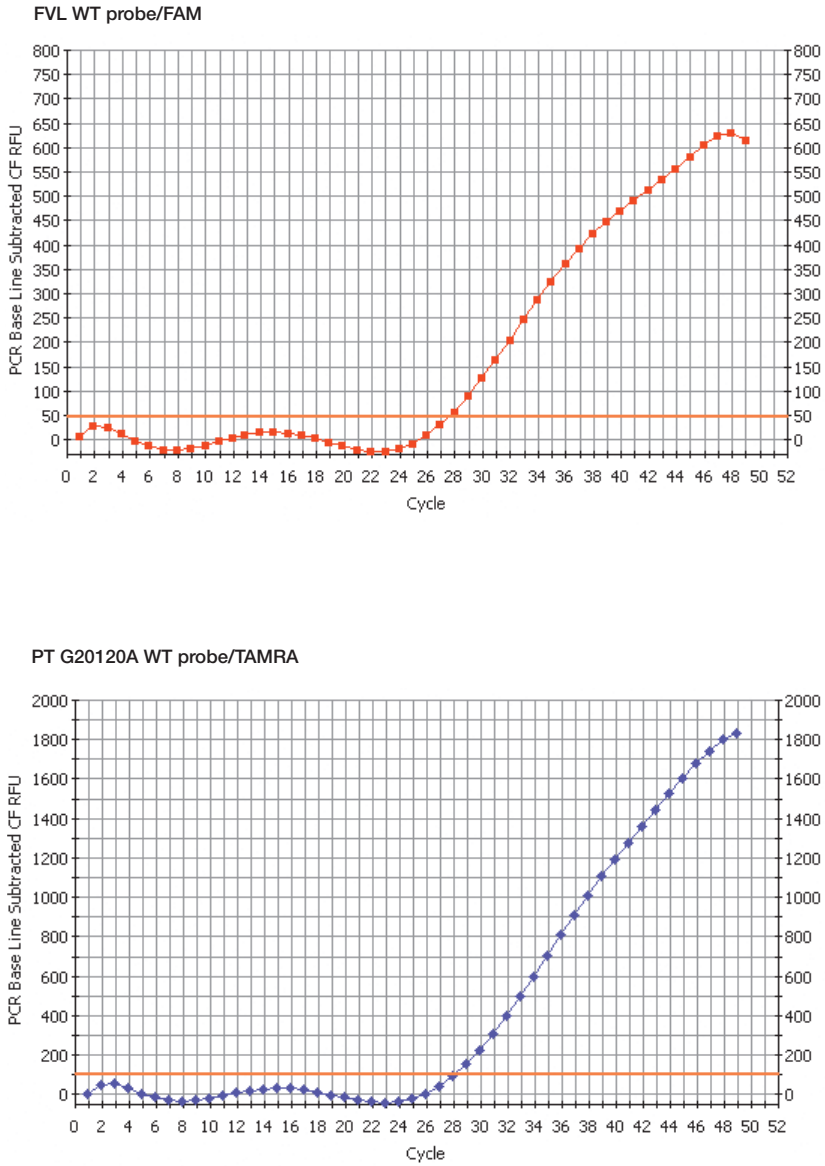
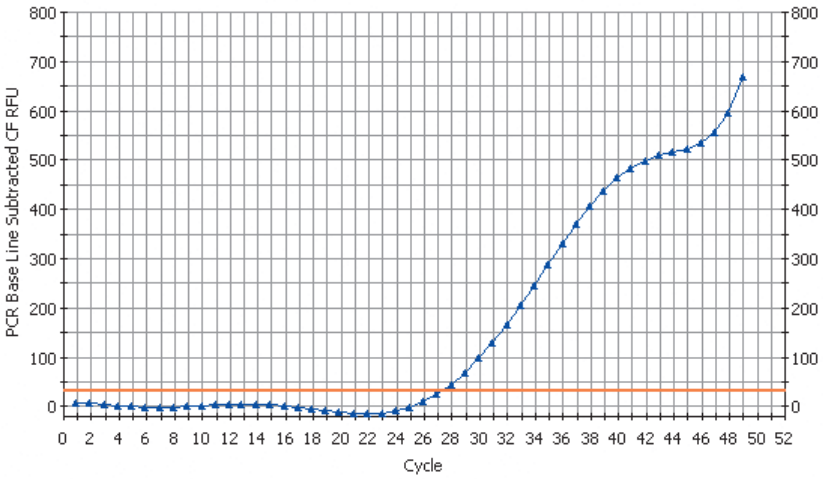


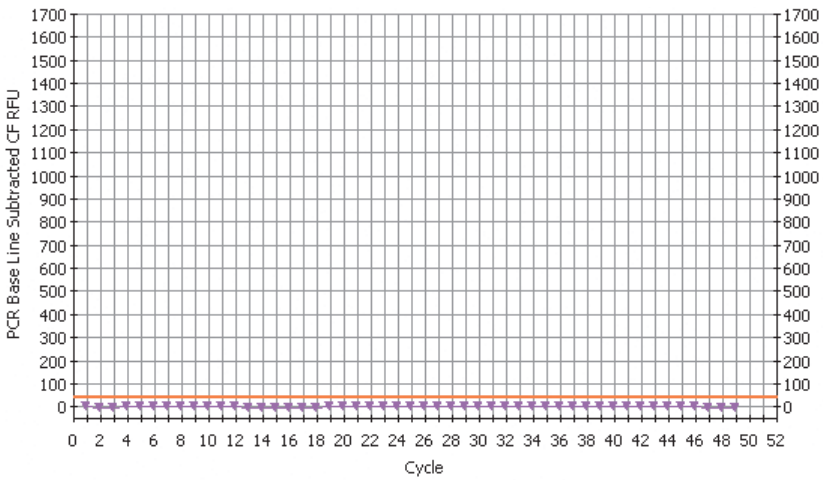

Fig. 6.5. Heterozygous genotype FVL G1691A.

**FVL mutant probe/TET**

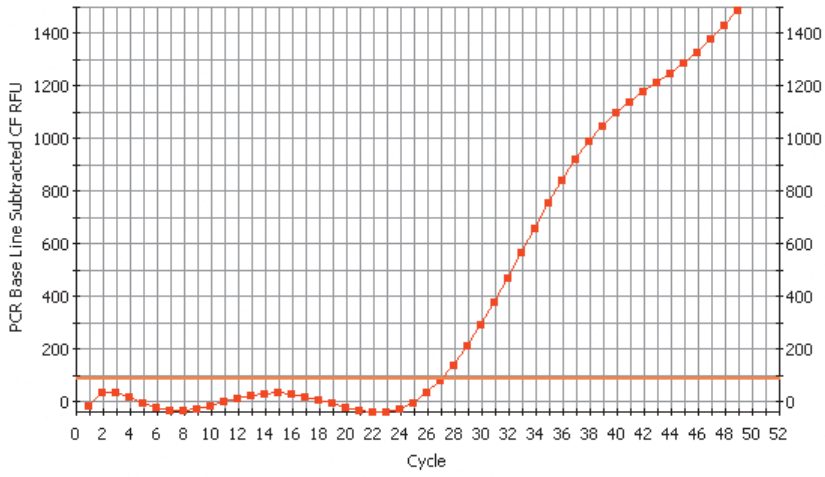



**PT G20210A mutant probe/Texas Red**



FVL WT probe/FAM




PT G20120A WT probe/TAMRA

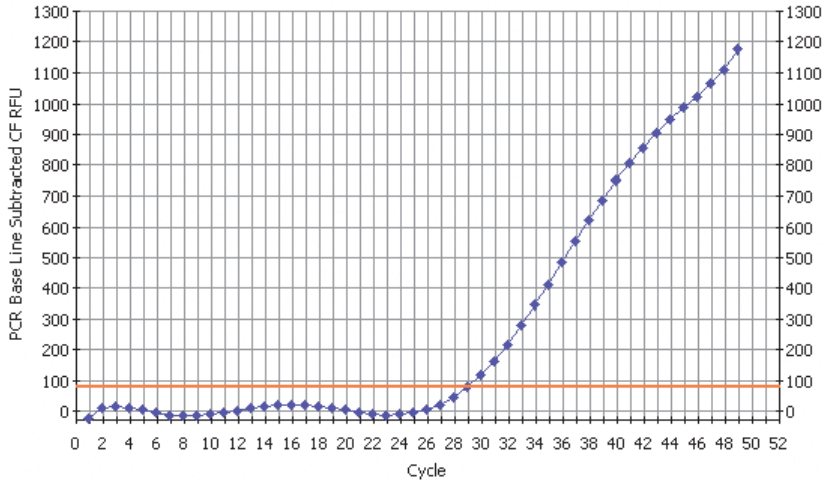
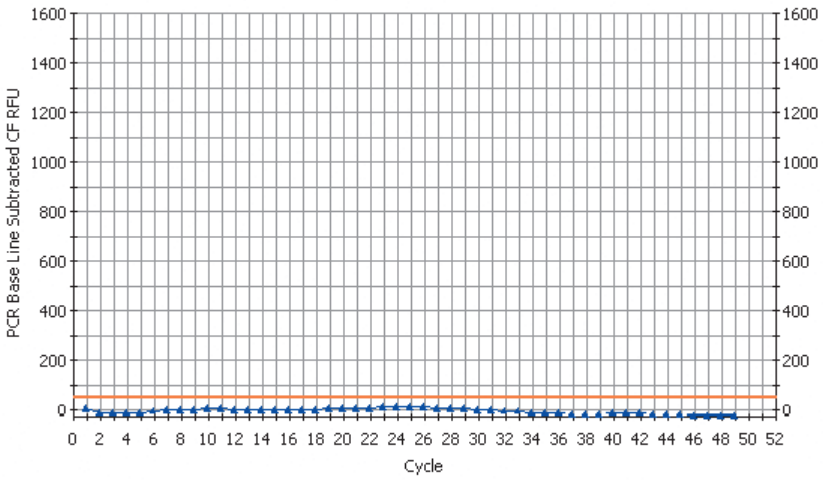


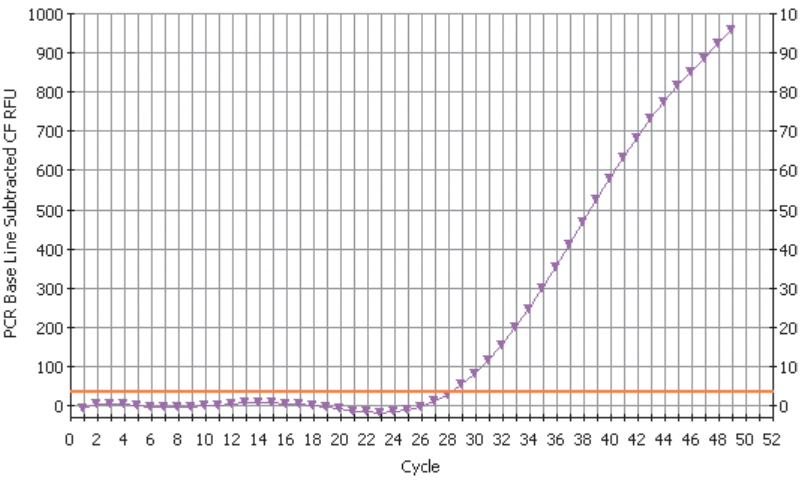

Fig. 6.6. Heterozygous genotype PT G20210A.

**FVL mutant probe/TET**

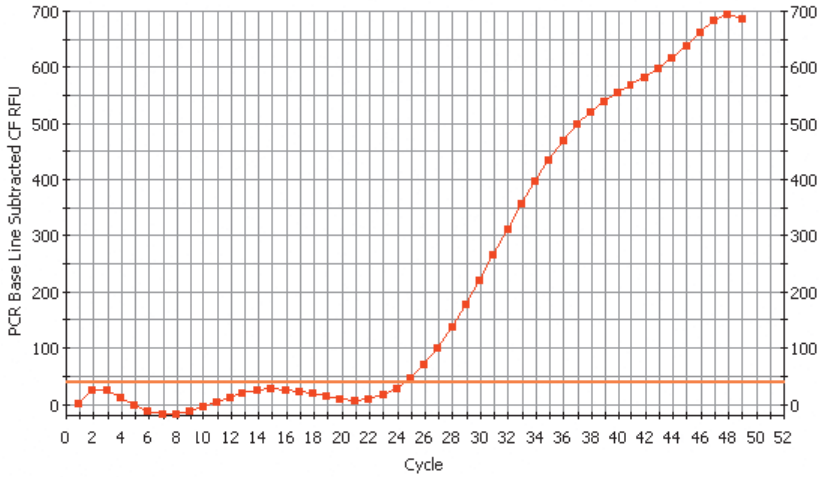



**PT G20210A mutant probe/Texas Red**



FVL WT probe/FAM




PT G20120A WT probe/TAMRA

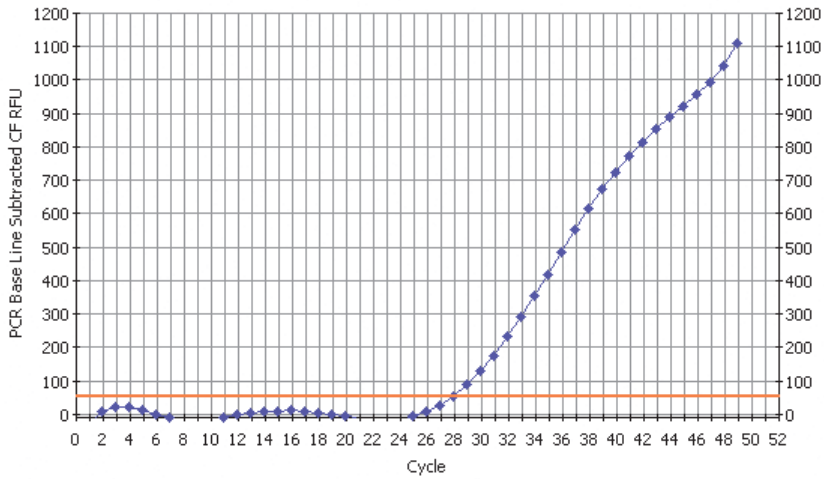
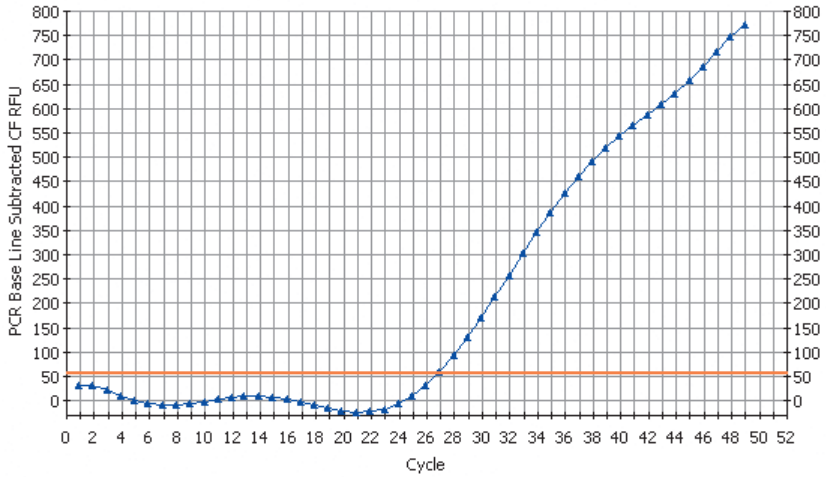


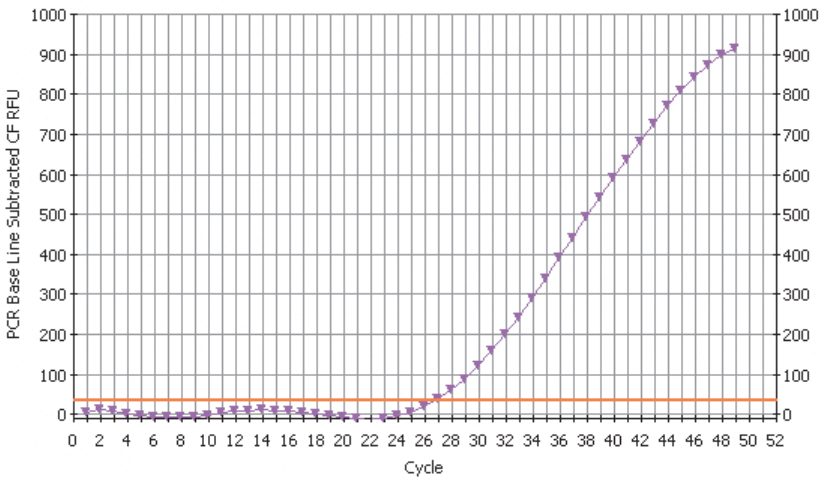

**Fig. 6.7. Compound heterozygous genotype FVL and PT G20210A.**

**FVL mutant probe/TET**




**PT G20210A mutant probe/Texas Red**



To examine the reproducibility of the four-color assay, six DNA samples from individuals with known genotypes (WT, FVL homozygous mutant, PT G20210A homozygous mutant, FVL heterozygous, PT G20210A heterozygous, and FVL/PT G20210A compound heterozygous) and a no-template control were tested in five experiments. For all cases, assays of the samples gave consistent and unambiguous genotypes.

## 6.7.2 Genotype Assignments

Genotypes can be assigned by analyzing the amplification plots generated for each allele-specific probe or by using the allelic discrimination analysis module available in the software of many real-time instruments, including the iCycler iQ, DNA Engine Opticon® 2, the Chromo4, and the iQ5 systems (see Product Guide). After the real-time PCR amplifications are finished, the iCycler iQ, iQ5 and Opticon Monitor™ software packages can assign genotypes using an algorithm that takes into account the signal from the assigned WT and homozygous mutant controls. These software packages can analyze either the final relative fluorescent units (RFU) or the $C_T$ values and graph the data as a scatter plot.

For the multicolor, multiplex assay described here, genotypes for each gene were assigned by the iCycler iQ software using the RFU mode and are shown on separate plots. Figure 6.8 shows the genotype assignments for the coagulation factor V gene, with four clearly defined clusters shown on the plot. The first cluster consists of samples with FAM signal values (x-axis) within 1,050–1,700 RFU and represents individuals with the WT genotype. The second cluster includes samples with TET signal values (y-axis) within 1,000–1,600 RFU and represents individuals

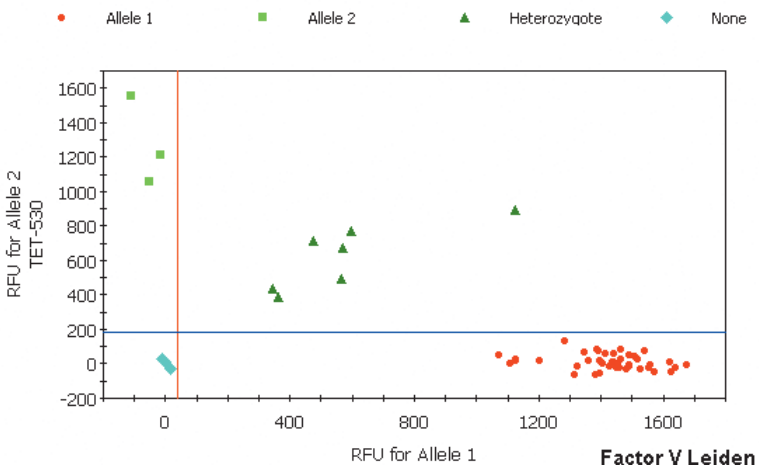



**Fig. 6.8. Coagulation factor V genotyping with the multicolor, multiplex TaqMan assay.**
The normalized fluorescence values of FAM (WT probe) vs. TET (FVL mutant probe) are shown.

with the FVL homozygous mutant genotype. The third cluster (FVL heterozygous individuals) includes samples with FAM and TET signal values within 300–1,100 RFU. The last cluster represents samples with very low RFU values for both FAM and TET; these are the no-template controls. Similarly, Figure 6.9 shows the PT genotypes in four well-defined clusters. The genotyping results generated by the multiplex four-color assay and analyzed by the software were in agreement with the results of an RFLP genotyping assay (data not shown).

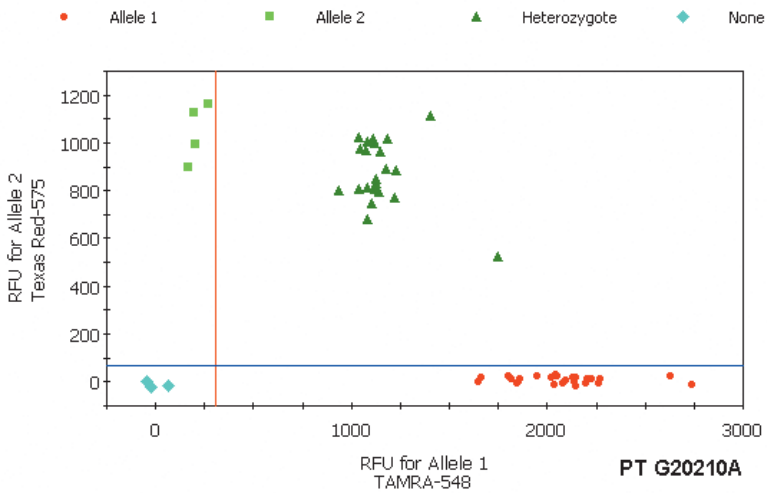



**Fig. 6.9. PT genotyping with the multicolor, multiplex TaqMan assay.** The normalized fluorescence values of TAMRA (WT probe) vs. Texas Red (PT mutant probe) are shown.

# Genetically Modified Organism (GMO) Detection

# 7. Genetically Modified Organism (GMO) Detection

GMOs are typically engineered to confer beneficial traits ranging from enhanced nutritional value to resistance to herbicides and pests. Soy, corn, and cotton are the most common genetically modified (GM) crops. Controversy over GMO-derived food has led some countries to require the disclosure and labeling of GMO content on foods. As a result, accurate and reliable methods to detect GMOs are needed.

Current methods to detect GMOs include PCR-based methods and enzyme-linked immunosorbent assays (ELISAs). Although ELISAs are relatively inexpensive and rapid, PCR-based methods offer the additional advantages of high sensitivity and accuracy, robustness, and the option to detect GMOs quantitatively as well as qualitatively. Real-time qPCR, for example, can be used to determine the percentage of GMO in a food sample.

Herbicide-resistant soy is an example of a GM food crop. Resistance to the herbicide glyphosate is conferred by introducing a transgene that restores an essential amino acid pathway disrupted by glyphosate. The transgene is a glyphosate-tolerant version of the enolpyruvylshikimate-3-phosphate synthase (epsps) gene from the soil bacterium *Agrobacterium* sp. strain *cp4*. Expression of *cp4* epsps in modified plants, such as Roundup Ready soybeans, confers glyphosate resistance, and thus allows the use of glyphosate on fields growing these GM crops.

The *cp4* epsps gene is expressed from the cauliflower mosaic virus (CaMV) 35S promoter. The CaMV 35S promoter is often used in GM crops because it has well-defined sequence requirements, is expressed in a broad range of tissues, and does not need any viral trans-acting factors. Because of its prevalent use in genetic modification of crops, the CaMV 35S promoter is a useful target for qPCR designed to detect a range of GMOs.

In this section, we provide protocols to detect glyphosate-resistant soy from food samples using real-time PCR. The advantages of the methods described here include the ability to test multiple samples, the need for only small amounts of starting material, applicability to raw and processed foods, and adaptability for other GM crops, such as corn, rice, and peanuts.

# 7.1 Experimental Design

Two real-time PCR methods for detecting and quantifying GM soy in foods are presented here. (See Section 2.2.2 for a discussion of real-time chemistries (SYBR Green I and TaqMan), and Section 2.2.1 for singleplex and multiplex considerations.) The first protocol is a one-color (singleplex) assay that uses SYBR Green I to detect a GMO-specific sequence (a transgene conferring herbicide resistance) and an endogenous reference sequence (soy lectin) in separate, parallel reactions. The second protocol is a two-color multiplex assay that uses differentially labeled TaqMan probes: one to detect the GMO-specific promoter (the CaMV 35S promoter) sequence and the other to detect an endogenous reference sequence (soy lectin), where both reactions are carried out in a single reaction vessel. An endogenous soy reference gene was used to normalize the amount of GM soy to the total amount of soy in the sample. The amount of GM soy was quantified by interpolation using a standard curve generated from a set of certified reference standards, as discussed in detail in the Data Analysis section below.

# 7.2 DNA Extraction and Sample Preparation for GMO Detection

Reference standards used were dried soybean powder containing mass fractions of 0–5% Roundup Ready soybean (Sigma). These reference standards were prepared by the manufacturer by mixing dried non-GM soybean powder with Roundup Ready soybean powder. The food samples tested (soy burger, soy dessert, and two brands of soy flour) were obtained from a supermarket.

Dry samples, the reference standards and the soy flour, were used directly in the DNA isolation procedure, whereas wet samples, the soy burger and the soy dessert, were mashed to a fine, homogeneous paste with a mortar and pestle.

For the real-time PCR assays described in Sections 7.3 and 7.4, the DNA templates were prepared from reference standards and food samples using the DNeasy plant mini kit (Qiagen) using methods described in the kit protocol. DNA concentrations were determined by spectrophotometry and the quality verified by electrophoresis on a 1% agarose gel. Generally, we obtained 1–2 µg of DNA from 55–100 mg of each food sample.

# 7.3 GM Soy Detection Using a Singleplex SYBR Green I qPCR Assay

After DNA was isolated from food and reference samples as described in Section 7.2, the fraction of glyphosate-resistant soy in soy-based food samples was determined using the singleplex SYBR Green I qPCR assay described in this section. This assay detects a GMO-specific sequence, which is from the *Agrobacterium cp4* epsps gene, and an endogenous genomic DNA control sequence from the soy lectin gene, which is expressed in both GM and unmodified soy. Since SYBR Green I fluorescence is not target-specific and cannot discriminate between the GMO-specific target and the endogenous soy target, amplification of these targets was performed in separate wells in parallel, singleplex reactions. (See Section 2.2.2 for a discussion of real-time chemistries (SYBR Green I), and Section 2.2.1 for singleplex and multiplex considerations.) The percent GMO, which is a fraction of the total soy content in the soy-based food samples, is determined by comparison to reference standards and can be quantified down to approximately 0.5% (w/w) concentration in this assay using these standards.

## 7.3.1 Reaction Components

To amplify the GMO-specific sequence (a 356 bp region of the *cp4* epsps gene), the primers used were: forward, 5'-TGG CGC CCA AAG CTT GCA TGG C-3' and reverse, 5'-CCC CAA GTT CCT AAA TCT TCA AGT-3'. To amplify the endogenous control, a 318 bp region of the soy lectin gene, which is expressed in both GM and non-GM soy, the primers were: forward, 5'-GAC GCT ATT GTG ACC TCC TC-3' and reverse, 5'-GAA AGT GTC AAG CTT AAC AGC GAC G-3'.

Although the components in the SYBR Green I assay described here were added individually, preformulated PCR master mixes containing buffer, DNA polymerase, dNTPs, and SYBR Green I dye, such as iQ™ SYBR® Green supermix, may also be used. Reaction components for the singleplex SYBR Green I assays described here and their final concentrations are listed below. Separate reactions were assembled to amplify the GMO-specific *cp4* epsps gene and the soy lectin endogenous control gene, using the respective primers listed above.

100 ng/µl DNA
250 nM of each primer
200 µM dNTPs
2 mM $MgCl_2$
1x $Mg^{2+}$-free buffer
0.5x SYBR Green I
0.02 U/µl DyNAzyme II DNA polymerase (Finnzymes)
Sterile, nuclease-free water to final volume of 20.0 µl

### 7.3.2 Cycling Protocol

Parallel amplification of GMO-specific *cp4* epsps and the soy lectin sequences were performed on the DNA Engine Opticon® 2 system using the protocol below:

1. 94°C, 3 min
2. 94°C, 10 sec
3. 60°C, 15 sec
4. 72°C, 20 sec
5. 86°C, 2 sec
6. Plate read
7. Go to step 2, 39 more times
8. 72°C, 10 min
9. Melt-curve analysis: 65°C to 98°C, 0.2°C/read, 1 sec hold
10. 72°C, 10 min

End

### 7.3.3 Data Analysis

Because the assay described here uses SYBR Green I, we verified that each target was specifically amplified (i.e., no nonspecific amplicons were produced) by melt-curve analysis, which showed a single peak for each amplicon (data not shown). See Section 2.2.2.1 for details on how melt-curve analysis can be used to analyze the specificity of a SYBR Green I-based qPCR assay.

The percent GM soy content of food samples was calculated in three steps: 1) GM soy was normalized to total soy content, yielding $\Delta C_T$, 2) a standard curve was generated using the reference standards ($\Delta C_T$ vs. log %GMO), and 3) the percent of GM soy in food samples was calculated by interpolation of the $\Delta C_T$ values of the normalized samples using the standard curve. Details for each step are given below:

1. GM soy was normalized to total soy by calculating the $\Delta\Delta C_T$ value. The $C_T$ from the control amplification (soy lectin) was subtracted from the $C_T$ of the GMO-specific amplification (epsps). For standards and samples that did not amplify a product with the epsps primers, the $\Delta C_T$ value was designated as not detected (ND).

$$\Delta C_T = C_T(\text{GMO epsps}) - C_T(\text{lectin})$$

The use of the $\Delta C_T$ assumes that the two reactions have similar efficiencies and that they proceed in a mutually independent manner since they were carried out in separate wells.

**Table 7.1. Calculation of $\Delta C_T = C_T(epsps) - C_T(lectin)$**

| Standard sample | $C_T$(epsps) | $C_T$(lectin) | $\Delta C_T$ |
|---|---|---|---|
| 0.0% GMO standard | ND | 19.68 | ND |
| 0.1% GMO standard | ND | 19.78 | ND |
| 0.5% GMO standard | 26.85 | 19.58 | 7.27 |
| 1.0% GMO standard | 25.62 | 19.52 | 6.10 |
| 2.0% GMO standard | 24.56 | 19.70 | 4.86 |
| 5.0% GMO standard | 22.94 | 19.48 | 3.46 |
| Soy dessert | 22.78 | 19.27 | 3.51 |
| Soy flour | ND | 18.13 | ND |
| Soy burger | 22.32 | 19.91 | 2.41 |

2. A standard curve was generated using the reference standards: the $\Delta C_T$ value of the reference standards were plotted on the x-axis and the log %GMO of the standards were plotted on the y-axis. Figure 7.1 shows the reference standards for 0–5% GMO. Data from the reference standards were fitted to a line using the Microsoft Excel chart function "Add Trendline". The function also returns the $R^2$ value, which indicates how well the linear equation fits the data.

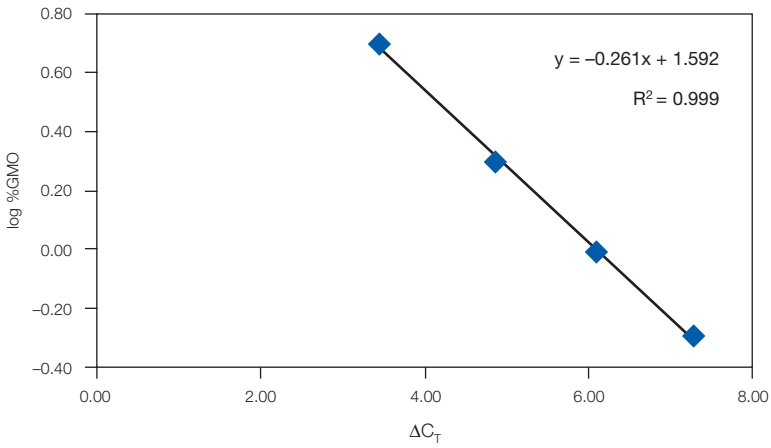



**Fig. 7.1. Standard curve generated from the $\Delta C_T$ values of the soy reference standards.** The log %GMO in each standard is shown. The $R^2$ value was 0.999.

**Table 7.2. Determination of percent GM soy.**

| Sample | $\Delta C_T$ | %GMO |
|---|---|---|
| Soy dessert | 2.1 | 4.40 |
| Soy flour* | ND | 0–0.5 |
| Soy burger* | 0.7 | >5 |

* These were outside the range of concentrations of the standards.

3. The percent of GM soy in test food samples was calculated by interpolation using the standard curve with the least-squares method using the Trend function in Excel software. In the Trend function, the following values were specified for the standards: log %GMO (the known y values), $\Delta C_T$ (the known x values), and the $\Delta C_T$ of the food sample for the new x. This function returned the value log %GMO of the food sample. The percent GMO soy content of the sample was then determined using the formula $10^{(\log \%GMO)}$.

# 7.4 GM Soy Detection Using a Multiplex TaqMan qPCR Assay

After DNA was isolated from food and reference samples as described in Section 7.2, the fraction of glyphosate-resistant soy (i.e., GM soy) in soy-based food samples was determined using another qPCR assay, the multiplex TaqMan assay described in this section. This assay detects the GMO-specific sequence (a sequence from CaMV 35S promoter) and an endogenous control sequence from the soy lectin gene, which is present in both GM and unmodified soy. The sequence-specificity of TaqMan probes allows the amplification and detection of more than one reaction in a single reaction vessel. In this single-tube assay, a FAM-labeled probe was used to detect the CaMV 35S promoter sequence, while a VIC-labeled probe was used to detect soy lectin. (See Section 2.2.2 for a discussion of real-time chemistries (TaqMan) and Section 2.2.1 for singleplex and multiplex considerations.) The percent GM soy, which is a fraction of the total soy content in the soy-based food samples, is determined by comparison to reference standards and can be quantified down to approximately 0.5% (w/w) concentration in this assay.

### 7.4.1 Reaction Components

Components for the multiplex TaqMan assay for GM soy detection described here are listed below. The primers, probes, dNTPs, $MgCl_2$, and buffer were from a preformulated kit (TaqMan GMO Soy 35S detection kit, Applied Biosystems). The GMO-specific CaMV 35S promoter was amplified using GMO-specific primers and detected by a FAM-labeled probe, while the soy endogenous control gene was amplified with lectin primers and detected with a VIC-labeled probe, in the same reaction tube:

2.0 µl DNA
17.5 µl GMO kit mix (Applied Biosystems)
0.5 µl AmpliTaq Gold (Applied Biosystems)
20.0 µl final volume

### 7.4.2 Cycling Protocol

Amplifications of the GMO-specific CaMV 35S promoter and the soy lectin sequences were performed on the DNA Engine Opticon 2 system using the protocol below:

1. 94°C, 9 min
2. 96°C, 20 sec
3. 60°C, 1 min
4. 72°C, 30 sec
5. Plate read
6. Go to step 2, 39 more times

End

### 7.4.3 Data Analysis

As described in Section 7.3.3, the percent GM soy content of food samples was calculated in three steps: 1) GM soy was normalized to total soy content, 2) a standard curve was generated using the reference standards ($\Delta C_T$ vs. log %GMO), and 3) the percent of soy in food samples that was genetically modified was calculated by interpolation of the $\Delta C_T$ values of the normalized samples using the standard curve. The analysis and results of the multiplex TaqMan assay are as follows:

1. GM soy was normalized to total soy by calculating the $\Delta C_T$ value. For standards and samples that did not amplify a product with the CaMV primers, the $\Delta C_T$ value was designated as “not detected” (ND).

$$\Delta C_T = \Delta C_T(\text{CaMV}) - \Delta C_T(\text{lectin})$$

**Table 7.3. Calculation of $\Delta C_T = C_T(epsps) - C_T(lectin)$**

| Standard or Sample | $C_T$(CaMV) | $C_T$(lectin) | $\Delta C_T$ |
|---|---|---|---|
| 0.0% GMO standard | ND | 24.40 | ND |
| 0.1% GMO standard | 35.96 | 24.30 | 11.66 |
| 0.5% GMO standard | 33.19 | 23.60 | 9.59 |
| 1.0% GMO standard | 32.12 | 23.60 | 8.52 |
| 2.0% GMO standard | 31.34 | 24.00 | 7.34 |
| 5.0% GMO standard | 29.76 | 23.80 | 5.96 |
| Soy burger | 27.22 | 21.85 | 5.37 |
| Soy flour | ND | 20.73 | ND |

2. Generate a standard curve using reference standards. Figure 7.2 shows the reference standards for 0–5% GMO.

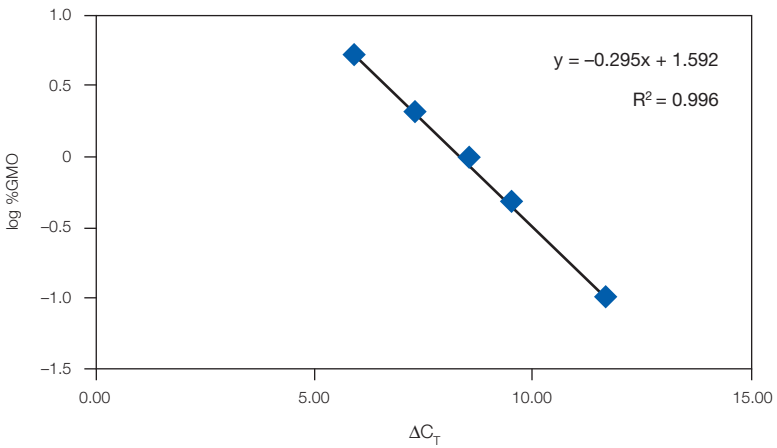



**Fig. 7.2. Standard curve generated from the $\Delta C_T$ values of the soy reference standards.** The log %GMO in each standard is shown. The $R^2$ value was 0.996.

3. Determine GMO content of food samples by interpolation using the standard curve.

**Table 7.4. Determination of percent GM soy.**

| Sample | $\Delta C_T$ | %GMO |
|---|---|---|
| Soy burger* | 4.41 | >5 |
| Soy flour* | ND | 0–0.1 |

* These were outside the range of concentrations of the standards.

# Product Guide

**Real-Time PCR Detection Systems**

| | |
|---|---|
| CFB-3120 | MiniOpticon™ Real-Time PCR System, includes optical housing, MJ Mini™ thermal cycler, analysis software |
| 170-9770 | MyiQ™ Single-Color Real-Time PCR Detection System, includes iCycler® base, optics module, software CD-ROM, 96-well optical reaction module, optical-quality 96-well PCR plates, Microseal® 'B' seals, communication cables, power cords, instructions |
| 170-9780 | iQ™5 Multicolor Real-Time PCR Detection System, includes iCycler base, optics module, software CD-ROM, 5 installed filter sets, 96-well reaction module, calibration solutions, optical-quality 96-well PCR plates, Microseal 'B' seals, communication cables, power cord, quick reference cards, instructions |
| CFB-3260G | Chromo4™ Four-Color Real-Time PCR Detector, includes optical housing, photonics shuttle, DNA Engine® thermal cycler, 96-well sample block, analysis software |

**Sample Preparation Reagents**

| | |
|---|---|
| 732-6800 | Aurum™ Total RNA 96 Kit, 2 x 96-well preps |
| 732-6820 | Aurum Total RNA Mini Kit, 50 preps |
| 732-6470 | Aurum Vacuum Manifold |
| 732-6830 | Aurum Total RNA Fatty and Fibrous Tissue Kit |

**Sample Assessment**

| | |
|---|---|
| 700-7001 | Experion™ System, 100–120 V, for RNA analysis, includes electrophoresis station, priming station, vortex station, software, USB2 cable, test chip, instructions (analysis kits sold separately) |
| 700-7002 | Experion System, 220–240 V, for RNA analysis, includes electrophoresis station, priming station, vortex station, software, USB2 cable, test chip, instructions (analysis kits sold separately) |
| 700-7103 | Experion RNA StdSens Analysis Kit for 10 Chips, includes 10 RNA StdSens chips, 1,250 µl RNA gel, 20 µl RNA StdSens stain, 20 µl RNA ladder, 900 µl RNA StdSens loading buffer, 2 spin filters |
| 700-7104 | Experion RNA StdSens Analysis Kit for 25 Chips, includes 25 RNA StdSens chips, 2 x 1,250 µl RNA gel, 2 x 20 µl RNA StdSens stain, 2 x 20 µl RNA ladder, 2 x 900 µl RNA StdSens loading buffer, 4 spin filters |

| | |
|---|---|
| 700-7105 | Experion RNA HighSens Analysis Kit for 10 Chips, includes 10 RNA HighSens chips, 1,250 µl RNA gel, 20 µl RNA HighSens stain, 20 µl RNA ladder, 900 µl RNA HighSens loading buffer, 100 µl RNA sensitivity enhancer, 2 spin filters |
| 700-7106 | Experion RNA HighSens Analysis Kit for 25 Chips, includes 25 RNA HighSens chips, 2 x 1,250 µl RNA gel, 2 x 20 µl RNA HighSens stain, 20 µl RNA ladder, 2 x 900 µl RNA HighSens loading buffer, 2 x 100 µl RNA sensitivity enhancer, 4 spin filters |
| 170-2402 | VersaFluor™ Fluorometer, 100/120/220 V, includes one standard cuvette holder, 100 standard disposable cuvettes, and a choice of one excitation and one emission filter |
| 170-2403 | VersaFluor Fluorometer System, 120 V, includes same as 170-2402 with a 120 V thermal printer and cable |
| 170-2404 | VersaFluor Fluorometer System, 100 V, includes same as 170-2402 with a 100 V thermal printer and cable |

**Reverse Transcription Kits**

| | |
|---|---|
| 170-8890 | iScript™ cDNA Synthesis Kit, 25 x 20 µl reactions, includes 5x iScript reaction mix, iScript enzyme, nuclease-free water |
| 170-8891 | iScript cDNA Synthesis Kit, 100 x 20 µl reactions |
| 170-8896 | iScript™ Select cDNA Synthesis Kit, 25 x 20 µl reactions, includes 5x iScript Select reaction mix, iScript reverse transcriptase, oligo(dT) mix, random primer mix, gene-specific primer (GSP) enhancer solution, nuclease-free water |
| 170-8897 | iScript Select cDNA Synthesis Kit, 100 x 20 µl reactions |
| 170-8892 | iScript One-Step RT-PCR Kit With SYBR® Green, 50 x 50 µl reactions, includes iScript reverse transcriptase for one-step RT-PCR, 2x SYBR Green RT-PCR reaction mix, nuclease-free water |
| 170-8893 | iScript™ One-Step RT-PCR Kit With SYBR® Green, 200 x 50 µl reactions |
| 170-8894 | iScript One-Step RT-PCR Kit for Probes, 50 reactions |
| 170-8895 | iScript One-Step RT-PCR Kit for Probes, 200 reactions |

**Real-Time Reagents**

| | |
|---|---|
| 170-8880 | iQ™ SYBR® Green Supermix, 100 x 50 µl reactions |
| 170-8882 | iQ SYBR Green Supermix, 500 x 50 µl reactions |
| 170-8884 | iQ SYBR Green Supermix, 1,000 x 50 µl reactions |
| 170-8885 | iQ SYBR Green Supermix, 2,000 x 50 µl reactions, 50 ml bottle |
| 170-8860 | iQ™ Supermix, 100 x 50 µl reactions |
| 170-8862 | iQ Supermix, 500 x 50 µl reactions |
| 170-8864 | iQ Supermix, 1,000 x 50 µl reactions |
| 170-8848 | iQ™ Multiplex Powermix, 50 x 50 µl reactions |
| 170-8849 | iQ Multiplex Powermix, 200 x 50 µl reactions |
| 170-8850 | iTaq™ SYBR® Green Supermix With ROX, 200 x 50 µl reactions |
| 170-8851 | iTaq SYBR Green Supermix With ROX, 500 x 50 µl reactions |
| 170-8852 | iTaq SYBR Green Supermix With ROX, 1,000 x 50 µl reactions |
| 170-8853 | iTaq SYBR Green Supermix With ROX, 2,000 x 50 µl reactions, 50 ml bottle |
| 170-8854 | iTaq™ Supermix With ROX, 200 x 50 µl reactions |
| 170-8855 | iTaq Supermix With ROX, 500 x 50 µl reactions |
| 170-8856 | iTaq Supermix With ROX, 1,000 x 50 µl reactions |
| 170-8857 | iTaq Supermix With ROX, 2,000 x 50 µl reactions, 50 ml bottle |
| 170-8858 | ROX Passive Reference Dye, 0.5 ml |

**Conventional PCR Reagents**

| | |
|---|---|
| 170-8870 | iTaq™ DNA Polymerase, 5 U/µl, includes 250 U polymerase, 1.25 ml 10x PCR buffer, 1.25 ml 50 mM $MgCl_2$ solution |
| 170-8875 | iTaq DNA Polymerase, 5 U/µl, includes 5,000 U polymerase, 25 ml 10x PCR buffer, 25 ml 50 mM $MgCl_2$ solution |
| 170-8874 | dNTP Mix, 200 µl premixed solution, contains 10 mM each dNTP (dATP, dCTP, dGTP, dTTP) |

**Plastics**

| | |
|---|---|
| MSB-1001 | Microseal 'B' Seals, clear adhesive, 100 |
| 223-9441 | 96-Well 0.2 ml Thin-Wall PCR Plates, 25 |
| MLL-4851 | Multiplate™ Low-Profile 48-Well Unskirted PCR Plates, 8 x 6 format, white, thin-wall polypropylene, 50 |
| MLL-4801 | Multiplate Low-Profile 48-Well Unskirted PCR Plates, 8 x 6 format, natural color, thin-wall polypropylene, 50 |

**Software**

| | |
|---|---|
| 170-8734 | Beacon Designer Probe/Primer Design Software, includes CD-ROM, quick guide, instructions |
| 170-9753 | iQ5 Optical System Software, version 2.0 |
| 170-9753SE01 | iQ5 Optical System Software, Security Edition, single-seat license |
| 170-9753SE05 | iQ5 Optical System Software, Security Edition, 5-seat license |
| 170-9753SE10 | iQ5 Optical System Software, Security Edition, 10-seat license |

**References**

Mathews DH et al., Expanded sequence dependence of thermodynamic parameters provides robust prediction of RNA secondary structure, J Mol Biol 288, 910–940 (1999)

Rozen S and Skaletsky HJ, Primer3 on the WWW for general users and for biologist programmers, pp 365–386 in Bioinformatics Methods and Protocols: Methods in Molecular Biology (Krawetz S and Misener S, eds), Humana Press, Totowa, NJ (2000); source code available at http://fokker.wi.mit.edu/primer3/

Zuker M et al., Algorithms and thermodynamics for RNA secondary structure prediction: a practical guide, pp 11–43 in RNA Biochemistry and Biotechnology (Barciszewski J and Clark BFC, eds), Kluwer Academic Publishers, Boston (1999)

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