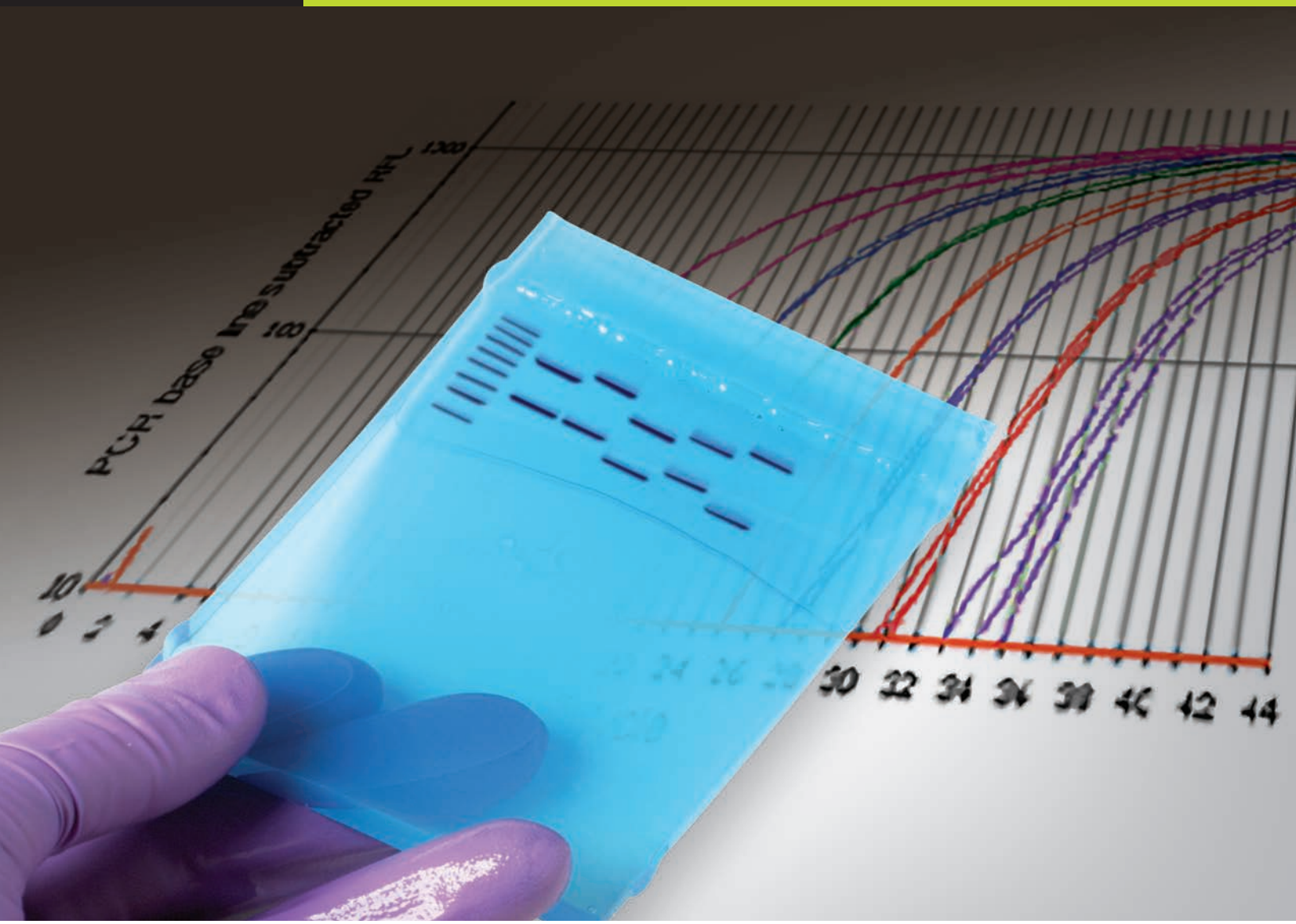
Application Note



Bio-Rad Explorer Crime Scene Investigator PCR Basics Kit:
A Real-Time PCR Extension

BIO-RAD

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This application note was developed in collaboration with Dr. David Palmer of Contra Costa Community College and Drs. Linnea Fletcher and Trish Phelps of Austin Community College. We'd like to thank each of them for the invaluable guidance and contribution each provided to this activity.

Introduction

This application note covers the use of the Crime Scene Investigator PCR Basics Kit as a foundation for a teaching lab on the use of real-time PCR technology.

Real-time PCR is an extremely important technology that is useful in food analysis, gene expression analysis, and many other applications in which the goal is to not only ask "what DNA is present" but also "how much." The Bio-Rad Explorer Crime Scene Investigator PCR Basics Kit is a tool for teaching students the principles of the polymerase chain reaction (PCR) and its use in forensic DNA analysis.

To teach the basics of real-time PCR in the classroom with the Crime Scene Investigator Kit reagents, simply substitute the Taq polymerase master mix with SsoAdvanced Universal SYBR® Green Supermix, use strip tubes and optical flat caps, and amplify the reactions on a real-time PCR instrument such as the CFX Opus or CFX Duet Real-Time PCR System. Although the Crime Scene Investigator PCR Basics Kit was developed for conventional PCR and end-point analysis of amplification of products by gel electrophoresis, this kit can be easily adapted for teaching the theory and technique of real-time PCR.

Given the robustness of the Crime Scene Investigator Kit and the expense of real-time PCR reagents, this kit is a good starting point for novices to become familiar with real-time PCR techniques. Since genotyping rather than quantitation is the goal for DNA fingerprinting, real-time PCR is not used in real-world short tandem repeat (STR) analysis that the Crime Scene Investigator Kit simulates. However, the real-time PCR application that this kit offers is a rich opportunity to learn the principles and techniques of real-time quantitative PCR. Additionally, the DNA fingerprints can still be investigated using gel electrophoresis and melt-curve analysis to demonstrate how real-time and conventional PCR can be complementary techniques.

Learning Objectives

At the end of this exercise, students will:

- Discover key differences between the goals of conventional PCR and real-time PCR analysis
- Analyze and evaluate real-time PCR results
- Learn the advantage of melt-curve analysis of the SYBR® Green I detection chemistry and compare this with final amplification products using agarose gel analysis
- Determine the accuracy and reliability of pipeting techniques by preparing duplicate or triplicate serial dilutions of template DNA
- Discover the sensitivity of PCR and how little template is required for detectable amplification results
- Understand how real-time PCR can quantitate the DNA in a sample
- Develop an understanding of the molecular basis of DNA amplification reactions using real-time thermal cyclers

Real-Time PCR Background Information for Instructors

Applications of Real-Time PCR

PCR has found so many applications in the biotechnology lab that it has been said: "PCR is to biology what petroleum is to transportation" (Pray 2004). Although there have been many ways that PCR has been adapted for the detection of specific nucleic acids in cells, real-time PCR is a widely used application of PCR in research and clinical diagnostic labs (Bustin et al. 2005, Kubista et al. 2006, Leutenegger 2001, Mackay 2004, Stevens 2003). The need for faster, more accurate, and more economical systems with a high throughput has fueled the popularity of real-time PCR.

Using genomic DNA as the template for amplification, real-time PCR can be used in infectious disease diagnostics to rapidly determine levels of specific pathogens in various tissues (Mackay 2004, Leutenegger 2001, Stevens 2003, Templeton et al. 2003). The molecular diagnostic lab also relies heavily on real-time PCR for detection of aneuploidies and the diagnosis of other genetic diseases (Gibson 2006, Jiang et al. 2004, Stevens 2003, Watson and Li 2005). In microbiology labs, real-time PCR can be used to detect and quantitate various microbial contaminants in environmental samples (Mackay 2004). This approach is especially invaluable in the analysis of microbes that are difficult to grow in culture.

Alternatively, using RNA as the template, reverse transcriptase can be used to generate DNA template for real-time PCR reactions, a strategy referred to as reverse transcription quantitative PCR (RT-qPCR), or as transcription-mediated amplification (TMA). This approach has become a valuable tool in the study of gene expression, where changes in transcription levels of target genes can be compared with those of a "housekeeping" gene that does not undergo changes in transcription. This technique has also become an important assay in the molecular diagnostic lab, where it can be used to determine the viral loading by retroviruses, or to diagnose disease using gene expression profiles (Bernard and Wittwer 2002, Bustin and Mueller 2005, Kubista et al. 2006, Saleh-Lakha et al. 2005, Wong and Medrano 2005).

Theory of Real-Time PCR

Conventional PCR does well to detect the presence of the DNA that the primer pair targets. Conventional PCR detects the amplified product (amplicon) by an end-point analysis — running the DNA on an agarose gel after the reactions are completed. If the target DNA sequence is not there, no amplicon will appear on the agarose gel. If as little as a single DNA molecule containing the target sequence is in the sample, 25–30 cycles of PCR amplification are sufficient to generate amplicons detectable via electrophoresis. Thus, conventional PCR makes a highly sensitive assay for specific DNA sequences, which is useful for the diagnosis of diseases, especially viral types. It is also a rapid, sensitive, and specific assay for microbes in environmental samples. Through the use of reverse transcriptase, conventional PCR has also become the standard for the detection of RNA targets, which is useful for gene expression analysis in research and clinical settings, as well as detection of viruses with RNA genomes. In this case, reverse transcriptase generates DNA from an RNA template, forming a template for PCR amplification.

Real-time PCR is based on the same principles as conventional PCR. The reaction requires both forward and reverse primers bracketing the target region, nucleotides, and a DNA polymerase. However, real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses — in "real-time." The difference is the addition of a fluorescence chemistry, which enables product amplification to be monitored throughout the entire real-time reaction using specialized thermal cyclers equipped with fluorescence detection modules. The measured fluorescence reflects the amount of amplified product in each cycle. 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 quantitative PCR (qPCR). The main advantage of using 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. Conventional PCR can at best be semi-quantitative and the methods to obtain quantitative data can be quite complicated. However, one advantage of conventional PCR is better determination of the sizes of the amplified PCR products using conventional gel electrophoresis. Therefore, separating the real-time PCR products on a gel following amplification allows the visualization and determination of the size of the amplified products.

How Real-Time PCR Works

To best understand how real-time PCR works, think of what is happening in a PCR reaction. During the first cycles of a PCR reaction, the amount of amplicon doubles. The amount of amplicon after each cycle then multiplies exponentially in proportion to the starting amount of template in the sample. At some point, this doubling slows as the amount of substrate, nucleotides, and primers are used up. The DNA polymerase also becomes less active after the prolonged heating within the thermal cycler. The loss of doubling efficiency results in a plateau effect, and the amount of amplicon produced with the later thermal cycles is no longer proportional to the amount of template DNA in the sample (Figure 1). After enough cycles, all amplicons reach a certain maximum concentration, regardless of the initial concentration of template DNA.

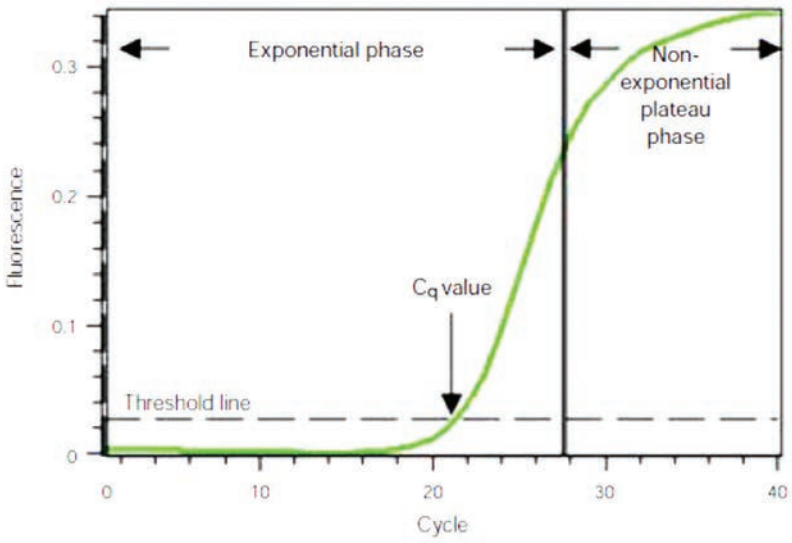

Figure 1. Amplification plot. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds and reaction components are consumed, the reaction slows and enters the plateau phase.

The key to determining the quantity of original template DNA present in a sample during amplification is to examine the initial thermal cycles before reaching the plateau region of amplification. To do this, the level of amplification is monitored continuously during thermal cycling. Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable (cycles 1–18 in Figure 1) even though PCR product accumulates exponentially. Eventually, enough amplified product accumulates to yield a detectable fluorescent signal. The cycle number at which this occurs is called the quantification cycle, or C_q[1]. Because the C_q 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 based on the known exponential function describing the reaction progress.

The C_q 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_q. 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_q. This relationship forms the basis for the quantitative aspect of real-time PCR.

[1] The cycle at which fluorescence from amplification exceeds background fluorescence has also been referred to as threshold cycle (C_t), crossing point (C_p), and take-off point (TOF) by various instrument manufacturers. Based on MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al. 2009 and 2010), the term is now standardized as the quantification cycle (C_q).

Optimizing a Real-Time Quantitative PCR Assay (qPCR)

Since real-time quantitation is based on the relationship between initial template amount and the C_q value obtained during amplification, an optimized qPCR assay is essential for accurate and reproducible quantitation of a sample. The hallmarks of an optimized qPCR assay are:

- Linear standard curve (R^2 > 0.980 or r > I-0.990I)
- Consistency across replicate reactions

A powerful way to determine whether your qPCR assay is optimized is to run a set of serial dilutions of template DNA and use the results to generate a standard curve. The template used for this purpose can be a target with known concentration (for example, nanograms of genomic DNA or copies of plasmid DNA) or a sample of unknown quantity (for example, cDNA). A standard curve is constructed by plotting the log of the starting quantity of template (or the dilution factor, for unknown quantities) against the C_q 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 2A. 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_q values of the curves). For example, with a 10-fold serial dilution of DNA, $2^n = 10$. Therefore, n = 3.32, and the C_q values should be separated by 3.32 cycles. Evenly spaced amplification curves will produce linear standard curves, as shown in Figure 2B. The equation and r values of the linear regression lines are shown above the plot.

The r or R^2 values of a standard curve represent how well the experimental data fit the regression line. This 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_q values between replicates will lower the r or R^2 values. You should strive for an r with an absolute value of >0.990 or an R^2 value >0.980 for your qPCR reactions.

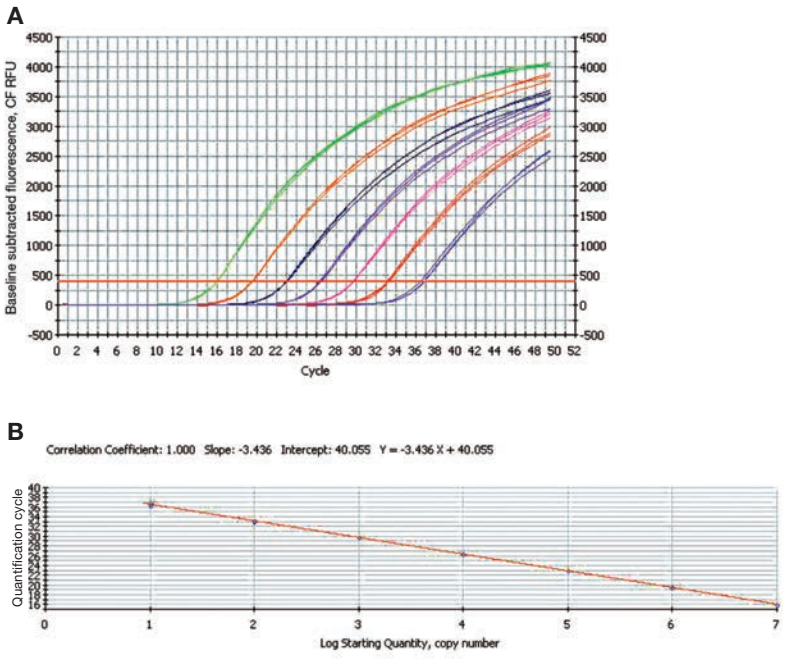

Figure 2. Generating a standard curve to assess reaction optimization. A standard curve was generated using a 10-fold dilution of a template. Each dilution was assayed in triplicate. **A.** Amplification curves of the dilution series. **B.** Standard curve with the C_q plotted against the log of the starting quantity of template for each dilution. The equation for the regression line and the r value (or correlation coefficient) are shown above the graph.

Chemistries for Monitoring Real-Time PCR

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:

- DNA-binding dyes (SYBR® Green I)
- Dye-labeled, sequence specific oligonucleotide primers or probes (molecular beacons, TaqMan assays, and hybridization probes)

Commonly used chemistries for real-time PCR include the DNA-binding dye SYBR® Green I and TaqMan hydrolysis probes.

SYBR® Green I is a DNA dye that binds non-discriminately to double-stranded DNA (dsDNA). SYBR® Green I exhibits minimal fluorescence when it is free in solution, but its fluorescence increases dramatically (up to 1000-fold) upon binding to dsDNA (Figure 3). As the PCR reaction progresses, the amplified product accumulates exponentially, more SYBR® Green I binds, and fluorescence increases.

The advantage of using SYBR® Green I is its simplicity. Though similar in action to ethidium bromide, SYBR® Green I does not interfere with DNA polymerases, so it can be added directly to a PCR reaction mixture. SYBR® Green I also has less background fluorescence than does ethidium bromide, is able to detect lower concentrations of dsDNA, and is not hazardous.

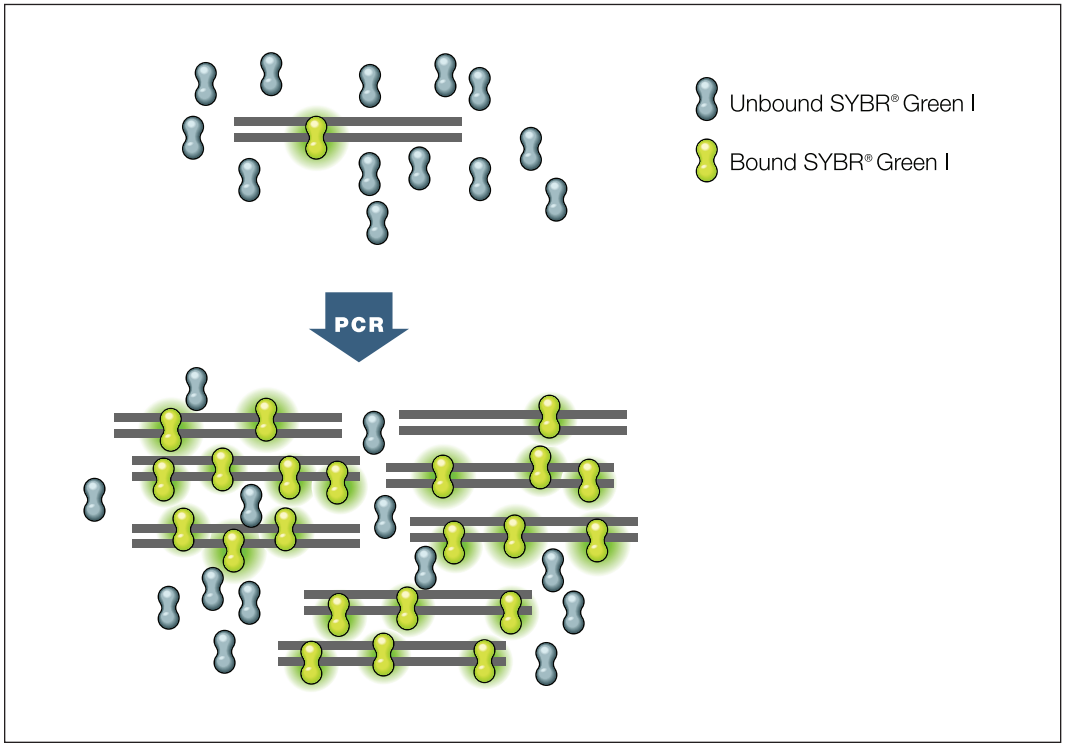

Figure 3. DNA-binding dyes in real-time PCR. Fluorescence dramatically increases when the dye molecules bind to double-stranded DNA.

Other advantages of using a dye that binds only to dsDNA include simple assay design (only two primers are needed; probe design is not necessary), lower initial cost (probes cost more), and the ability to perform a melt-curve analysis to check the specificity of the amplification reaction.

The main disadvantage to the use of SYBR® Green I is its nonspecificity. Since it will bind to any dsDNA, you cannot distinguish between the amplification of target DNA and the amplification of primer-dimers. Also, non-target sequences that are amplified will appear in a SYBR® Green I fluorescence curve and will be indistinguishable from amplification of target sequence. For this reason, when using SYBR® Green I, it is prudent to verify that target DNA is being amplified, for example by running an agarose gel of the reaction products (conventional PCR). Alternatively, post-amplification melt-curve analysis can be performed on the real-time PCR instrument to distinguish reaction products and analyze reaction specificity, eliminating the need for agarose gel analysis of reaction products.

Melt-Curve Analysis

The principle of the melt-curve analysis is that the temperature is increased from a low temperature (where all sequences are annealed) to a high temperature, causing strand dissociation, also called melting. As the dsDNA melts, SYBR® Green I is released and a decrease in fluorescence is observed. Two factors are important in melting temperatures: the size of the double-stranded DNA and the GC content. The higher the GC content and the larger the strand size, the higher the melting temperature will be. By comparing the melt temperatures of known amplicons, the presence of an extra non-target amplicon or primer-dimers is easily detected.

In a typical melt-curve, the fluorescence intensity is plotted against the temperature. The fluorescence decreases as the temperature increases and the dsDNA dissociates. There are two distinct stages to the curve: the rapid loss of fluorescence as the DNA begins to melt and the slower loss of fluorescence as the last of the dsDNA disassociates. Software can also plot the negative first derivative of the rate of change of fluorescence (in relative fluorescence units, or RFU) vs. temperature (-d(RFU)/dT). 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 4. 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 bands in lanes 2 and 3 on the gel.

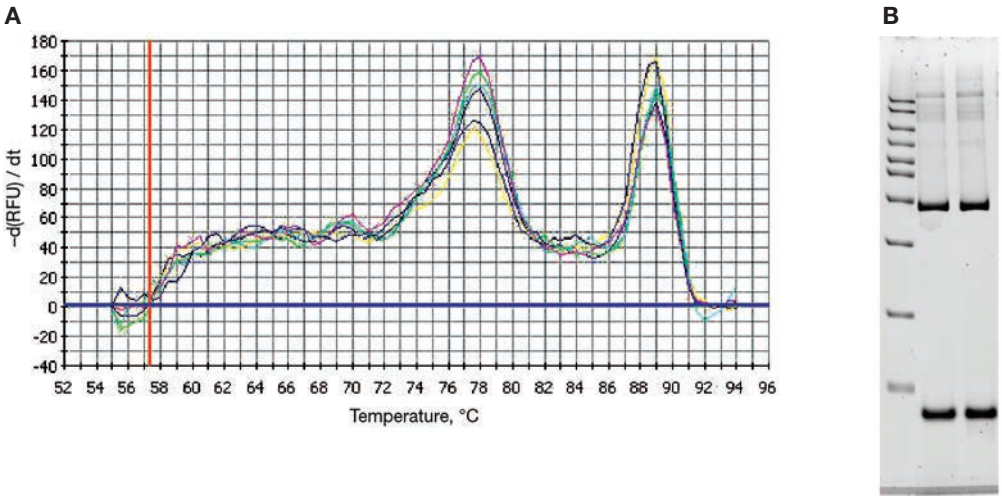

Figure 4. Melt-curve analysis of reaction product from a SYBR® Green I assay. The melt-curve analysis function of real-time instruments can be used to distinguish specific products from non-specific products. **A.** The negative first derivative of the change in fluorescence is plotted as a function of 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 appear as separate bands in the gel.

Special Precautions

It is imperative that best laboratory practices are followed when performing real-time PCR experiments. Given the extreme sensitivity of PCR, extra precautions must be taken to avoid cross contamination of template sources in equipment and reagents. Make sure to use a fresh pipet tip at each pipeting step. To avoid contamination of the micropipets themselves, the use of filtered tips is strongly advised. Gloves should be worn while performing a PCR experiment and they should be changed frequently to avoid cross contamination of DNA. Work areas should also be kept DNA-free. Note that ethanol is not an effective way to clean your work area. DNA is not soluble in ethanol and therefore ethanol does a poor job of removing DNA. A 10% bleach solution is probably the best approach as it will hydrolyze as well as dissolve the DNA. A consumer pump spray, like Formula 409 or Fantastik, can be an effective alternative to applying bleach to affected surfaces. In addition, screwcap tubes prevent spraying of your precious sample when you open the lid and help reduce contamination of gloved fingers when you open the tube.

Many people cannot pipet 2 µl reproducibly. It is difficult to tell when the volume is incorrect because of a loose tip or a worn and unreliable pipetor. One can visualize 5 µl in the tip and can usually tell if the volume is incorrect. Using pooled master mixes for replicate sample tubes can improve the assay's reproducibility by avoiding multiple pipeting steps and the necessity of pipeting small volumes. After transferring into a solution, rinse the pipet tip by gently pumping up and down into the solution several times to mix the sample and make the transfer more quantitative. Remember to vortex and spin samples down with a centrifuge before PCR, especially after thawing frozen samples. Finally, because real-time PCR relies on the optical detection of fluorescence coming from the PCR reaction itself, care must be taken to avoid introducing bubbles or foam into the PCR tubes before beginning the reaction.

Experimental Protocol

Materials and Methods

The Crime Scene Investigator PCR Basics Kit is designed to simulate real-world forensic DNA analysis at a single locus: amplified suspect DNA is compared to amplified crime scene DNA and the results are compared on an agarose gel. This kit can be extended to demonstrate real-time PCR in two different labs. Each may be performed separately or run in sequence for greater depth of understanding and increased hands-on experience.

Laboratory 1 preserves the original intent of the Crime Scene Investigator Kit. It allows students to observe real-time PCR as the reactions progress and allows the students to amplify and compare suspect versus crime scene DNA samples using agarose gel electrophorsis.

Laboratory 2 uses the primers and pre-made DNA samples in the Crime Scene Investigator Kit to explore relative quantification and melt-curve analysis.

Laboratory 1: Run the Full Crime Scene Investigator Kit Protocol on a Real-Time PCR Instrument

Introductory Level

Purpose

To carry out the Crime Scene Investigator protocol while viewing the PCR reactions on a real-time PCR instrument.

Laboratory 1 involves running the Crime Scene Investigator PCR Kit on a real-time PCR instrument, substituting a master mix with fluorescent dye (SsoAdvanced Universal SYBR® Green Supermix) for the master mix that is included with the kit. As the PCR reactions progress, the SYBR® Green I dye in the supermix binds with the double-stranded DNA generated by the PCR process. With every cycle in the PCR reaction, the amount of DNA produced doubles and more and more fluorescence will be generated. Eventually, the reactions fluoresce to the point that they can be detected by the instrument; they then will continue to increase in fluorescence every cycle. Ultimately, when the PCR reactions run out of reagents the fluorescence will no longer increase. Limiting reagents may include nucleotides, primers, template DNA, DNA polymerase, and SYBR® Green I fluorescent dye.

Materials

- Crime Scene Investigator PCR Basics Kit (1662600EDU)
- Small DNA Electrophoresis Reagent Pack (1660450EDU)
- SsoAdvanced Universal SYBR® Green Supermix (1725270EDU)
- PCR-grade water (proteomics-grade water is also acceptable, 1632091EDU)
- PCR tube strips (TLS0851EDU)
- Optical flat caps (TCS0803EDU)

Additional required items

- A real-time PCR instrument such the CFX Opus or CFX Duet Real-Time PCR System
- Agarose gel electrophoresis equipment
- Equipment as described in the Crime Scene Investigator PCR Basics Kit manual

Method

This experiment is carried out in principle the same as the regular protocol in the Crime Scene Investigator Kit manual but requires a few modifications.

1. Program the real-time PCR instrument as described below prior to the class or as a demonstration.
2. Directly substitute the 2x SsoAdvanced Universal SYSBR® Green Supermix for the PCR master mix described in manual.
3. Plan on 25 µl reactions instead of 40 µl reactions (for example, 12.5 µl of template and 12.5 µl of 2x supermix plus primers (MMP))
4. In addition to setting up the PCR reactions with the crime scene and suspect's DNA samples, include a negative control — a reaction with no DNA template (NTC- no template control) (12.5 ul of sterile water and 12.5 ul of MMP).
5. Do not use the 0.2 ml PCR tubes provided in the kit. PCR tube strips with optical flat caps must be used instead. Provide students with a strip of 6 tubes and 6 caps. Ensure the students do not label the tubes on the caps since most instruments read the fluorescence through the tube caps.
6. Ensure the students set up their reactions in the exact manner in which you have programmed the plate setup for the instrument (see below).
7. Run the PCR reactions on the real time PCR instrument.
8. View the PCR reactions in real time as they progress during the cycling.
9. Connect the real-time PCR instrument and computer to a projector for easier student viewing.
10. Once complete, add 6 µl of Orange G loading dye to the samples and separate the PCR products on a 3% agarose gel as described in the Crime Scene Investigator PCR Basics Kit manual.
11. Analyze the results.

Programming the Real-Time PCR Instrument

The real-time instrument should be programmed by the instructor prior to the class (possibly as a demonstration, if appropriate). Use the manual and software provided with the instrument to perform the setup according to the recommendations below. Due to the complexity of real-time PCR instruments, there are a lot of choices with regard to changing the parameters of the protocol. If a parameter is not specified below, use the instrument's default settings and consult the instruction manual or the instrument's technical support group for additional advice. For advice on using a Bio-Rad real-time PCR system, call 1-800-4BIO-RAD in the U.S. or contact your local Bio-Rad office.

Plate Setup

See Figure 5 for a suggestion on plate setup. It is essential that the students are aware that the correct placement of their samples in the thermal cycler is vital to the interpretation of their final results. Most instruments allow you to save the plate setup to use with other classes. To identify and orient the PCR strips, label the side of each PCR tube in the strip with an indelible marker. Do not write on the caps since that will interfere with the fluorescence reading.

	1	2	3	4	5	6
A	NTC	CS	A	B	C	D
B	NTC	CS	A	B	C	D
C	NTC	CS	A	B	C	D
D	NTC	CS	A	B	C	D
E	NTC	CS	A	B	C	D
F	NTC	CS	A	B	C	D
G	NTC	CS	A	B	C	D
H	NTC	CS	A	B	C	D

Figure 5. Plate setup. NTC (no template control); CS (crime scene DNA sample); A (suspect A DNA sample); B (suspect B DNA sample); C (suspect C DNA sample); and D (suspect D DNA sample).

The dye used in this experiment is SYBR® Green I, so if you are using a multicolor real-time PCR system, select this dye in your plate setup. Alternatively, you may identify the wells as containing FAM dye, since it is detected at the same wavelength as SYBR® Green I.

Programming the Protocol

Program the real-time thermal cycler with the following protocol:

The reaction volume is 25 µl.
The lid temperature should be 95–100°C.

Cycle 1: 94°C for 2 min — Initial denaturation of DNA

Cycle 2: 94°C for 30 sec — Denaturation
52°C for 30 sec — Annealing
72°C for 1 min — Extension — collect data after this step

Repeat Cycle 2 for 40 cycles

Cycle 3: Melt-curve analysis. Program the instrument to heat the samples from 55°C to 95°C in increments of 0.5°C and have the instrument collect data (or read the samples) after 10 seconds for each incremental step. Alternatively, use the instrument's default settings for the melt-curve data collection.

Save the protocol to the instrument's library.

Laboratory 1 Expected Results

Figure 6 shows the fluorescence of the PCR reactions as a function of cycle number, observed in real time.

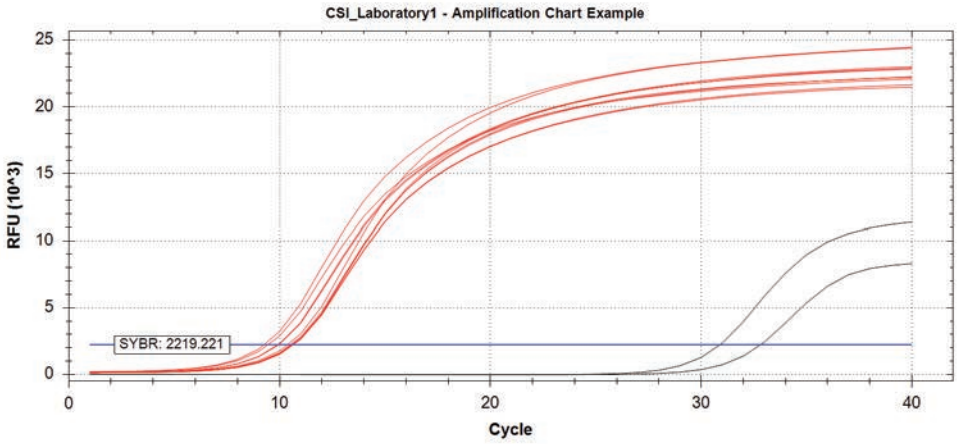

Figure 6. PCR Amplification plot. Color key: red = CSI kit reactions, black = no template control. RFU = relative fluorescence units.

The graph clearly shows that the Crime Scene Investigator Kit PCR reactions began to amplify to significant levels after cycle number 5. By cycle number 20, the curves have entered the plateau phase. This indicates that the PCR process had run to completion. Interestingly, samples without any template DNA (the "no template control") began to show amplification late in the PCR cycling (after cycle 30). This is due to the formation of PCR primer-dimers, which are the result of the primers themselves serving as DNA templates. Viewed in real time, as the PCR run is progressing, the graph above is a visually dramatic demonstration of how DNA is amplified in the thermal cycler.

Melt-curve analysis shows the temperature at which a double-stranded amplicon dissociates into single-stranded DNA, thus releasing the SYBR® Green I and resulting in a decrease in fluorescence. Different amplicons dissociate at different temperatures based upon the length of the amplicon and the GC content. Melt-curve analysis for this data set potentially show five different amplicons plus a primer-dimer peak. To reduce this complexity, view the melt curves for a single reaction or for a set of reactions from a single DNA sample. It should be possible to identify specific peaks representing the two amplicons amplified in the reaction that should match the gel electrophoresis results. It is also possible to overlay the crime scene sample data with each of the suspects (one suspect at a time) to see whether there is a match. Do you have as much confidence in the match from the melt-curve data as from the agarose gel electrophoresis data?

When the PCR products from the real-time reactions are separated on an agarose gel, the band patterns typically expected from the kit are easily visualized.

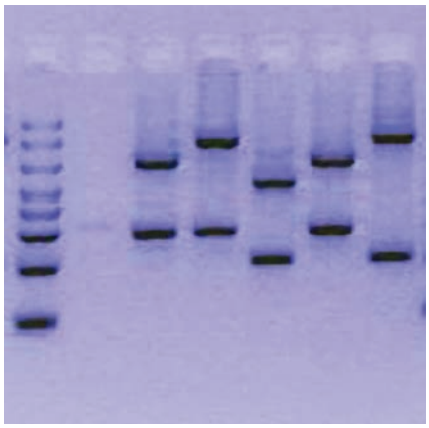

Figure 7. Real-time PCR products separated on a 3% agarose gel. Lanes from left to right: allele ladder, NTC, CS, Suspects A, B, C, and D.

In Figure 7, the crime scene DNA clearly matches the band pattern for the DNA obtained from suspect C, and clearly differs from the DNA obtained from the other three suspects. The agarose gel results indicate that carrying out the Crime Scene Investigator Kit on a real-time PCR instrument, using real-time PCR reagents, in no way adversely affects the quality of the PCR results when viewed on a gel.

Laboratory 2: Use the Crime Scene Kit DNA and Primers to Demonstrate Quantitative PCR (qPCR)

Advanced Level

Purpose

To demonstrate the rapid and quantitative nature of real-time PCR by evaluating a dilution series with real-time PCR reagents and instrumentation.

Materials

- Crime Scene Investigator PCR Basics Kit (1662600EDU)
- Small DNA Electrophoresis Reagent Pack (1660450EDU)
- SsoAdvanced Universal SYBR® Green Supermix (1725270EDU)
- PCR-grade water (proteomics-grade water is also acceptable, 1632091EDU)
- PCR tube strips (TLS0851EDU)
- Optical flat caps (TCS0803EDU)

Additional Required Items for qPCR

- A real-time PCR instrument (such the CFX Opus or CFX Duet Real-Time PCR System)
- Agarose gel electrophoresis equipment
- Equipment as described in the Crime Scene Investigator PCR Basics Kit manual

Instructor's Overview

- The Crime Scene Investigator Kit has sufficient reagents for 80–100, 25 µl real-time PCR reactions
- The protocol is written assuming a real-time PCR instrument with 48 wells is available; if your real-time instrument has 96 wells, the breadth of the experiment can be expanded with students setting up additional reactions either from different templates or an expanded dilution series permitting construction of a standard curve
- Each student team will amplify duplicate reactions of three dilutions of one of the suspect, crime scene DNA templates, or a no template control. Depending on the number of student teams, some templates may be amplified by more than one team
- Do not use the 0.2 ml PCR tubes provided in the kit. PCR tube strips with optical flat caps must be used instead. Provide students with a strip of 8 tubes and 8 caps. Ensure the students do not label the tubes on the caps since most instruments read the fluorescence through the tube caps
- Reagents can either be aliquoted or taken by students from a master stock depending on the instructor's preference

Programming the Real-Time PCR Instrument

The real-time instrument should be programmed by the instructor prior to the class (possibly as a demonstration, if appropriate). Use the manual and software provided with the instrument to perform the setup according to the recommendations below. Due to the complexity of real-time PCR instruments there are a lot of choices with regard to adjusting the parameters of the protocol. If a parameter is not specified below use the instrument's default settings and consult the instruction manual or the instrument's technical support group for additional advice. For advice on the CFX Opus or CFX Duet System, call 1(800)4-BIO-RAD in the US or contact your local Bio-Rad office.

Plate Setup

See Figure 8 for a suggestion on plate setup. It is essential that the students are aware that the correct placement of their samples in the thermal cycler is vital to the interpretation of their final results. Most instruments will allow you to save the plate setup to use with other classes. To identify and orient the PCR strips, label the side of each PCR tube in the strip with an indelible marker. Do not write on the caps since that will interfere with the fluorescence reading.

	1	2	3	4	5	6
A	A1	B1	C1	D1	CS1	
B	A1	B1	C1	D1	CS1	
C	A2	B2	C2	D2	CS2	
D	A2	B2	C2	D2	CS2	
E	A3	B3	C3	D3	CS3	
F	A3	B3	C3	D3	CS3	
G	A4	B4	C4	D4	CS4	
H	A4	B4	C4	D4	CS4	

Figure 8. Plate setup. A (suspect A DNA sample); B (suspect B DNA sample); C (suspect C DNA sample); D (suspect D DNA sample); and CS (crime scene DNA sample) in duplicate at various dilutions. Numbers 1–3 correspond to the three dilution factors, while number 4 corresponds to the no template control.

The dye used in this experiment is SYBR® Green I, so if you are using a multicolor real-time PCR system (such as a CFX Connect Real-Time PCR Detection System, iQ5 Real-Time PCR Detection System, CFX Opus System or CFX Duet System), be sure to select this dye in your plate setup. Alternatively, you may identify the wells as containing FAM dye, since it is detected at the same wavelength as SYBR® Green I.

Programming the Protocol

Program the thermal cycler with the following fast PCR protocol.

The reaction volume will be 25 µl.
The lid temperature should be 95–100°C.

Cycle 1:	94°C for 2 min	Initial denaturation of DNA
Cycle 2:	94°C for 10 sec	Denaturation
	52°C for 30 sec	Annealing and Extension — collect data
	Repeat Cycle 2 for 40 cycles.	

Cycle 3: Melt-curve analysis. Program the instrument to heat the samples from 55°C to 95°C in increments of 0.5°C and have the instrument collect data (or read the samples) after 10 seconds for each incremental step. Alternatively, use the instrument's default settings for the melt-curve analysis.

Save the protocol to the instrument's library.

Note: This is a fast PCR protocol that may preferentially amplify the smaller suspect target DNA or have decreased amplification compared to Laboratory 1. You will, however, still be able to see amplification of all smaller bands with this quicker protocol. If desired, use the protocol in Lab 1 (page 10) for a more robust amplification of all the alleles.

Student Protocol

Thaw Tubes

1. On ice, thaw the DNA samples and primers from the Crime Scene Investigator Kit and the SsoAdvanced Universal SYBR® Green Supermix.

Prepare Serial Dilutions of DNA

2. The crime scene and suspect DNA will be diluted 100, 10,000, and 1,000,000 fold. Each student team will prepare four 1.5 ml microfuge tubes, labeled with the DNA template name (either A, B, C, D, or CS) and dilution reference.

   Example for DNA Sample Suspect A:
   Suspect A #1 — 1/100 dilution
   Suspect A #2 — 1/10,000 dilution
   Suspect A #3 — 1/1,000,000 dilution
   Suspect A #4 — no template control

3. Add 990 µl sterile distilled water to each of the above tubes.

   Add 10 µl of concentrated template DNA to tube labeled #1. Mix tube thoroughly by vortexing for at least 10 seconds or by flicking tube at least 20 times.
   Transfer 10 µl diluted DNA from tube #1 to tube #2. Mix thoroughly.
   Transfer 10 µl diluted DNA from tube #2 to tube #3. Mix thoroughly.
   Tube #4 will have no DNA template added.

Prepare PCR Tubes

4. Obtain an eight-well PCR tube strip. You will prepare duplicate PCR reactions. Label each pair of tubes with the template name and the dilution reference, and be sure to only write on the sides of the tubes. For example, the eight-tube strip for the group(s) analyzing Suspect A would have each tube labeled as follows: A1; A1; A2; A2; A3; A3; A4; A4. Ensure the reactions are set up in the exact manner programmed into the real-time PCR instrument.

5. Add 12.5 µl of template DNA from each tube of your dilution series to the pair of corresponding PCR tubes.

Prepare PCR Master Mix

6. Label a fresh 1.5 ml microtube "MM".

7. Pipet 110 µl of SsoAdvanced Universal SYBR® Green Supermix to the MM tube.

8. Pipet 2.2 µl of the Crime Scene Investigator Kit primers (blue) to the MM tube. Mix thoroughly by vortexing for 10 seconds or flicking the tube 20 times. Spin down the tube contents.

Add Master Mix to PCR Tubes

9. Add 12.5 µl of master mix to each of the 8 PCR reactions, using a fresh pipet tip each time. Mix the reaction gently by slowly pipeting up and down several times, without withdrawing the pipet tip. Remember that this PCR will be detected optically, so be careful to avoid introducing bubbles into the PCR reaction.

10. Cap tubes using the optical flat caps. Gently tap tubes on bench or use a microfuge with an adaptor to ensure the mixture is at the bottom of the tubes.

Run PCR Reactions

11. Run the PCR reactions on the real-time PCR instrument.

12. View the PCR reactions in real time as they progress during the cycling.

13. Connect the real-time PCR instrument and computer to a projector for easier student viewing.

14. (Optional) Once complete, add 6 µl of Orange G loading dye to the samples and run the PCR products out on a 3% agarose gel as described in the Crime Scene Investigator Kit manual. Please note that using the two-step fast cycling protocol will amplify only the 200 and 300 bp alleles and thus will not permit matching the crime scene DNA with the suspect.

15. Analyze the results.

Laboratory 2 Expected Results

The real-time PCR reactions will show up as curves similar to those shown in the following graphs. The 1/100 diluted DNA, being most concentrated, will have curves that appear “earliest”, for example, around cycle 10. Those diluted 1/10,000 fold will appear later, perhaps around cycle 20. Finally, those samples diluted 1/1,000,000 will appear last, after cycle number 25. The no template control will contain PCR primer-dimers and will begin to amplify around cycle 30 (or possibly not at all).

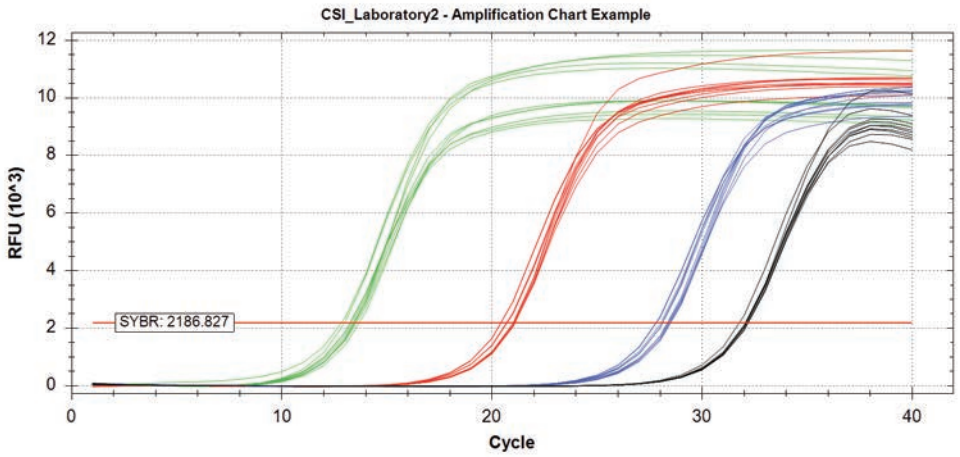

Figure 9. PCR Amplification plot. Color key: Green=100X, Red=10,000X, Blue=1,000,000X , Black=NTC. RFU=relative fluorescence units.

The above quantitation graph clearly demonstrates the quantitative nature of real-time PCR. Samples containing higher concentrations of starting DNA result in curves that rise earlier. The point at which these curves cross the quantification cycle (commonly called the C_q value) directly relates to the starting quantity of template. These C_q values can subsequently be used to accurately quantitate DNA concentrations. For example, since PCR involves a doubling of the amount of DNA every cycle, curves with C_q values that differ by 3 cycles would represent 2^3 = an 8-fold difference in starting template concentration.

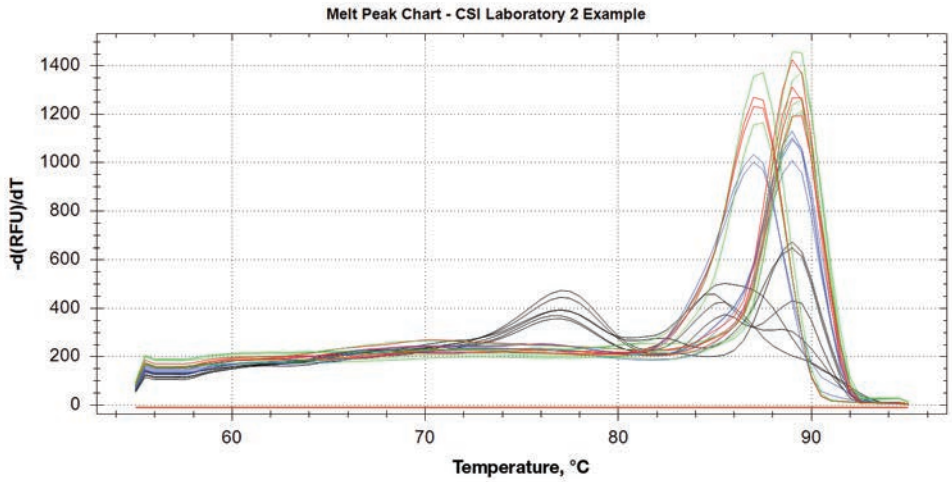

Figure 10. Melt-curve plot. Color key: Green=100X, Red=10,000X, Blue=1,000,000X, Black=NTC.

The melt-curve analysis for the Crime Scene Investigator Kit, run under a fast real-time protocol, will typically reveal three distinct peaks. However, each reaction will typically yield one or two peaks: one representing the major bands amplified and a smaller primer-dimer peak. Different PCR products often have different melting temperatures (the temperature at which the DNA strands separate or melt). In this case, the peak close to 75°C represents the primer-dimers and the two peaks above 85°C represent the larger amplicons from different suspect DNA samples in the Crime Scene Investigator Kit.

Conclusions

The Crime Scene Investigator Kit, which comes with DNA template and primers of known high quality, provides an excellent foundation for classroom demonstration of real-time PCR.

Vivid Demonstration of PCR Reactions as They Occur

Because the Bio-Rad SsoAdvanced Universal SYBR® Green Supermix can directly replace the PCR master mix included with the Crime Scene Investigator Kit, simply substituting the mixes and adding a no-template control allows the educator to run the whole Crime Scene Investigator Kit on a real-time PCR instrument. This vividly demonstrates the principles of DNA amplification as the run progresses, since the students can directly see the curves (representing DNA quantity in the reaction) start to climb, then climb very quickly, and finally plateau. In addition, the PCR products can then be removed from the real-time instrument and viewed on an agarose gel, completing the Crime Scene Investigator Kit and identifying the “suspect”.

How Real-Time Quantitative PCR Measures the Amount of DNA

Further modifying the Crime Scene Investigator Kit, as suggested in Laboratory 2, the components of the Crime Scene Investigator Kit can be turned into a dilution series. This dilution series, when examined by real-time PCR analysis, clearly demonstrates how the amount of starting DNA template directly affects the position of the PCR amplification curves — the basic principle of quantitative PCR.

Faster PCR

In addition, Laboratory 2 can be run with a “fast” PCR protocol, allowing the entire PCR lab to run very quickly (curves will easily be visible within an hour).

Measuring Pipeting Variation

If the educator decides to have replicates of the PCR reactions, then the curves between matching replicates can be compared. If the curves aren’t exactly in the same position between replicates, this may indicate variations in pipeting error between students.

The Importance of Proper Experimental Controls

The Laboratory 2 serial dilution experiment clearly indicates that the lowest dilutions of DNA (one million fold diluted from the DNA that comes with the kit) require many more cycles to amplify a significant amount of product. So many cycles, in fact, that the primer-dimers (that occur naturally in samples without any DNA template at all) amplify at about the same point. Therefore, this experiment shows that positive results in real-time PCR do not necessarily mean that there was any starting template DNA.

Melt-Curve Analysis

Finally, the melt-curve analysis that is easily incorporated at the end of a real-time PCR reaction can be used to discuss the dynamics of DNA. At some temperatures DNA is single-stranded, at others it is double-stranded, and the difference between the two configurations is critical for the operation of PCR. Also, melt-curve analysis generates visible “peaks” that can be used to determine what type of PCR products have been produced (primer-dimers or amplicons of different sizes).

Glossary

Agarose gel — A gel, made of an uncharged agarose polymer, that is typically used to separate nucleic acids and other biomolecules by size via electrophoresis.

Amplicon — PCR product; the DNA produced by amplification in a PCR reaction.

Amplification — An increase in the amount of a DNA sequence resulting from the polymerase chain reaction (PCR).

Amplification efficiency — A measure of how closely the majority of PCR cycles in a given run approximate perfect doubling of product. The amplification efficiency, E, is calculated from the slope of the standard curve using the formula E = 10–1/slope. The percent amplification efficiency is calculated using the formula %E = (E–1) x 100%, where 100% efficiency is an indicator of a robust assay.

Amplification plot — The graphical representation of changes in relative fluorescence units (RFU) per real-time PCR cycle.

Annealing step — A PCR step in which the reaction is cooled to allow primers to bind to the denatured template.

Baseline — Fluorescence emitted during the initial cycles of real-time PCR, during which the changes in fluorescent amplification signal are not detectable in a given sample.

cDNA — Complementary DNA. A DNA sequence that is synthesized from mRNA template by reverse transcription. The cDNA sequence is complementary to the mRNA template sequence.

Coefficient of determination (R^2) — A statistical measure of the degree of linear or nonlinear association between two variables, which indicates the strength of that association. Represents how well the experimental data fit the regression line. In the case of a simple linear regression, R^2 is the square of the correlation coefficient (r).

Correlation coefficient (r) — A statistical measure of the degree of linear relationship between two variables that indicates the strength of that relationship. Represents how well the experimental data fit the regression line. A coefficient of 1 means a perfect linear relationship.

C_q — See quantification cycle.

Cycle — One round of denaturation, annealing, and extension steps in a PCR protocol.

Denaturation step — A PCR step in which the reaction is heated to break the hydrogen bonds that hold double-stranded DNA together, thus separating the DNA into single-stranded molecules. Typical denaturing temperatures are 92–95°C.

DNA — Deoxyribonucleic acid; strands of linked deoxyribonucleotides that contain the genetic information of cells. By convention, the sequence of nucleotide bases (A, T, G, and C) is written in the 5′ to 3′ direction.

dNTP — Abbreviation for a deoxynucleoside 5′-triphosphate when the exact deoxynucleotide (typically dATP, dTTP, dGTP, or dCTP) is unspecified or unknown.

dsDNA — A double-stranded DNA.

Efficiency — See amplification efficiency.

Exponential phase — The period of an amplification reaction during which the product accumulates exponentially, approximately doubling with every cycle. During this phase, the amplification plot can be approximated by an exponential equation and a plot of log RFU vs. cycle number can be fit by a line.

Expression	See gene expression.
Extension step	A PCR step in which the temperature is set to allow DNA polymerase to extend the primer by adding nucleotides complementary to the template sequence.
FAM	5- or 6-carboxyfluorescein, a fluorescent molecule commonly used to monitor the amplification of target in probe-based real-time PCR assays.
Fluorescence	Light of a specific range of wavelengths that is emitted from a molecule previously excited by energy of a different range of wavelengths. Each fluorescent molecule has characteristic excitation and emission spectra.
Gene expression	The regulated transcription of DNA, generating mRNA. PCR techniques allow the detection of relative gene expression in a sample by amplifying the cDNA generated from purified mRNA.
Gradient	In a thermal cycler, a controlled, incremental temperature differential across a reaction block.
Housekeeping gene	Genes that are continuously expressed in virtually all cells due to the constant requirement of the gene product for basic cellular function. Their expression is typically not affected by experimental treatments. These genes are often used as reference genes in relative quantification.
Inhibitor	In PCR, a substance that prevents efficient amplification.
Marker	See molecular weight marker.
Molecular weight marker	A DNA fragment of known size used as a standard for comparison when estimating the size of unknown DNA fragments following gel electrophoresis. A series of molecular weight markers is sometimes called a ladder.
Master mix	A concentrated mixture of reaction components that can easily and consistently be diluted with water, primers, and template for use in a PCR.
Melt-curve	A plot of fluorescence vs. sample temperature used to determine the melting temperature (T_m) of a population of DNA molecules, to distinguish specific products from nonspecific products in real-time PCR. To create the curve, one must use a reporter chemistry that distinguishes dsDNA from single-stranded DNA (e.g., SYBR® Green I), and measure fluorescence while incrementally increasing the sample temperature.
mRNA	Messenger RNA. An RNA molecule that is translated into a protein in the cell. For gene expression studies, mRNA can be reverse-transcribed into cDNA, which in turn serves as the initial template for PCR.
Oligonucleotide	A relatively short, single-stranded sequence of nucleotides, synthesized artificially and used as a probe or primer to bind a complementary sequence. Also called “oligo”.
PCR	See polymerase chain reaction.
Plateau phase	The nonexponential phase of PCR that occurs after many cycles, when the rate of amplification decreases.
Polymerase chain reaction (PCR)	A technique that uses a series of denaturation, annealing, and extension steps to copy (amplify) specific DNA or cDNA sequences.

Primer	An oligonucleotide that binds to a DNA template and serves as the starting point for DNA strand elongation during the extension step of PCR. To amplify a specific target, two primers are designed, one complementary to each strand of the dsDNA, such that the two bracket the target of interest.
Primer-dimer	An artifact or nonspecific product, composed of annealed primers, that forms when there is homology within or between PCR primers.
Quantification cycle (C_q)	In real-time PCR, the cycle number (in the exponential phase) at which enough amplified product has accumulated to yield a detectable fluorescent signal (i.e., to cross the threshold fluorescence level). The value is used to accurately and reliably measure the number of cycles required to detect dsDNA amplification in the sample.
qPCR	Quantitative PCR, a technique that uses fluorescently labeled molecules to track the accumulation of amplified products with each cycle of PCR.
r	See correlation coefficient.
R^2	See coefficient of determination.
Real-time PCR	A technique that uses fluorescently labeled molecules to track the accumulation of amplified products with each cycle of PCR.
Reference gene	A gene whose expression level is used for normalization in relative quantification. See housekeeping gene.
Relative quantification	A type of real-time PCR data analysis used to calculate differences in a target concentration across different samples. The technique uses a reference, which is expected to have the same concentration in every sample, to normalize the target concentration in each sample.
Reverse transcriptase	An RNA-dependent DNA polymerase. Used in RT-PCR to transcribe an mRNA sequence into cDNA.
Reverse transcription	The process of transcribing mRNA to cDNA using reverse transcriptase.
RFU	Relative fluorescence units; a unit of fluorescence intensity measured by a real-time PCR detection system.
RNA	Ribonucleic acid; a nucleic acid, transcribed from DNA, that plays an important role in translating genes into proteins.
RT	See reverse transcription.
RT-PCR	Reverse transcription PCR. A PCR technique in which reverse transcriptase synthesizes cDNA from RNA template, and the cDNA is subsequently amplified by PCR.
Standard curve	A quantitative research tool used to determine the concentration or copy number of an unknown substance. In PCR, the C_q values obtained when amplifying a series of known nucleic acid quantities is plotted, and the graph is used to estimate the quantity of unknown nucleic acid sequences.
SYBR® Green I	A fluorescent molecule that binds nonspecifically to dsDNA. When free in solution, SYBR® Green I exhibits little fluorescence, but its fluorescence increases up to 1,000-fold when it binds to dsDNA. SYBR® Green I is commonly used for nonspecific detection of PCR products in real-time PCR assays.

Taq polymerase A thermally stable DNA polymerase used in PCR to amplify nucleic acid. This polymerase also exhibits 5' exonuclease activity, which cleaves the reporter from the 5' end of a TaqMan probe during amplification.

Target The specific nucleotide sequence that is to be amplified during the PCR reaction.

Template The gDNA or cDNA sample used in a PCR study or the nucleic acid sequence that serves as the pattern for the synthesis of its complementary nucleic acid strand.

T_m Melting temperature. The temperature at which 50% of DNA of a given sequence is denatured into single strands. The melting temperature is heavily dependent upon the DNA length and GC content.

References

Bernard PS and Wittwer CT (2002) Real-time PCR technology for cancer diagnostics. Clin Chem 48, 1178–1185.

Bustin SA et al. (2005) Quantitative real-time RT-PCR—a perspective. J Mol Endrocrinology 34, 597–601.

Bustin SA and Mueller R (2005) Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. Clin Sci 109, 365–379.

Bustin SA et al. (2009) The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55, 611–622.

Bustin SA et al. (2010) MIQE précis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. BMC Mol Biol 11, 74–78.

Bustin SA et al. (2011) Primer sequence disclosure: A clarification of the MIQE Guidelines. Clin Chem 57, 919–921.

Gibson NJ (2006) The use of real-time PCR methods in DNA sequence variation analysis. Clin Chim Acta 363, 32–47.

Jiang YT et al. (2004) Genotyping Parkinson disease-associated mitochondrial polymorphisms. Clin Med Res 2, 99–106.

Kubista M et al. (2006) The real-time polymerase chain reaction. Mol Aspects Med 27, 95–125.

Leutenegger CM (2001) The real-time TaqMan PCR and applications in veterinary medicine. Vet Sci Tomorrow 1, 1–15.

Mackay IM (2004) Real-time PCR in the microbiology laboratory. Microbiol Infect 10, 190–212.

Pray PA (2004) Consider the Cycler. The Scientist, 34–37.

Saleh-Lakha S et al. (2005) Microbial gene expression in soil: methods, applications, and challenges. J Microbiol Methods 63, 1-19.

Stevens CD (2003) Clinical Immunology and Serology: A Laboratory Perspective. 2nd Ed. F.A. Davis Co. Philadelphia.

Templeton KE et al. (2003) Evaluation of real-time PCR for detection of and discrimination between *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella homesii* for clinical diagnosis. J Clin Microb 41, 4121–4126.

Watson DE and Li B (2005) TaqMan applications in genetic and molecular toxicology. Int J Toxicol 24, 139–45.

Wong ML and Medrano JF (2005) Real-time PCR for mRNA quantification. BioTechniques 39, 484.

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