

**Bio-Rad Explorer[™]
Crime Scene Investigator
PCR Basics[™] Kit:**
A Real-Time PCR Extension



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Introduction

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

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. Real-time PCR is an extremely important technology, 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”.

To teach the basics of real-time PCR in the classroom with the Crime Scene Investigator kit reagents simply substitute the Taq polymerase mastermix with iQ™ SYBR® Green supermix, use strip tubes and optical flat caps, and amplify the reactions on a real-time Bio-Rad PCR instrument such as the CFX Connect™ Real-Time PCR Detection System. Although the Crime Scene Investigator PCR Basics kit was developed for conventional PCR and end-point analysis of amplification products by gel electrophoresis, this kit can be easily adapted for instruction in real-time PCR.

Given the robustness of the Crime Scene Investigator kit and the expense of the 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 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 showing how real-time and conventional PCR can be complimentary 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 becoming the most widely used application of PCR in the research lab for genomic and gene expression analysis, and is rapidly establishing itself as a technique in the clinical diagnostic lab (Bustin et al, 2005; Kubista, 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, 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, 2004; Stevens, 2003; Watson, 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 quantitative reverse transcriptase PCR (qRT-PCR), 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 various mRNAs can be compared with those of a gene that does not undergo changes in transcription, most often these tend to be the so-called “housekeeping genes”. 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 by expression profiles (Bernard, 2002; Bustin & Mueller, 2005; Kubista, 2006; Saleh-Lakha, 2005; Wong, 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 that contains the target DNA sequence is in the sample, the amplification by 25-30 cycles is sufficient to generate detectable amplicons via electrophoresis. Thus, conventional PCR makes a highly sensitive assay for specific DNA sequence, which is useful for the diagnosis of diseases, especially viral types. It is also a rapid, highly 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, useful for analysis of gene expression in research and medical diagnosis. In this case, reverse transcriptase generates DNA from an RNA template, forming a template for the PCR polymerase 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 (amplicon), nucleotides, and a DNA polymerase such as *Taq*. 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 Bio-Rad 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 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

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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. 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 become 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.

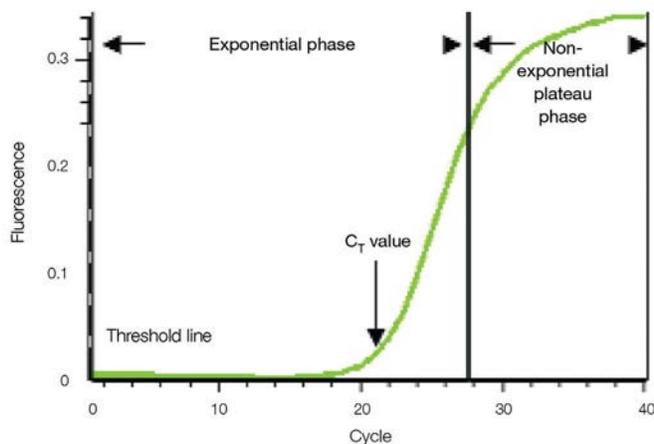


Figure 1. Amplification plot. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds and reaction components are consumed, the reaction slows and enters the plateau phase.

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 the 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 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.

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Optimizing a Real-Time Quantitative PCR Assay (qPCR)

Since real-time quantitation 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 quantitation of your particular sample. The hallmarks of an optimized qPCR assay are:

- Linear standard curve ($R^2 > 0.980$ or $r > | -0.990 |$)
- 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_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 2A. If perfect doubling occurs with each amplification cycle, the spacing of the fluorescence curves will be determined by the equation $2^n = \text{dilution factor}$, 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 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 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 and R^2 value >0.980 for your qPCR reactions.

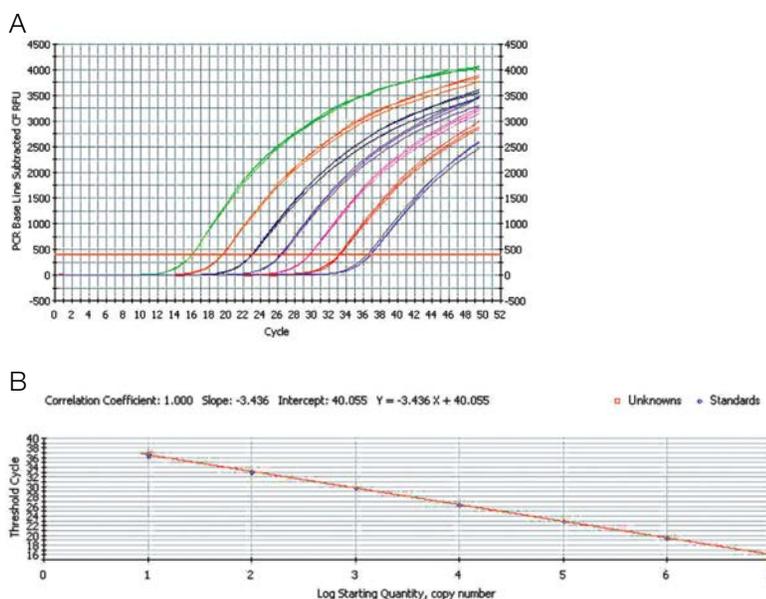


Figure 2. Generating a standard curve to assess reaction optimization. A standard curve was generated using a 10-fold dilution of a template amplified on the iCycler IQ[®] real-time system. Each dilution was assayed in triplicate. **A.** Amplification curves of the dilution series. **B.** Standard curve 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.

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)

The most commonly used chemistries for real-time PCR are the DNA-binding dye SYBR® Green I and TaqMan hydrolysis probe. We provide an overview of SYBR® Green I fluorescence chemistry below. For more information regarding TaqMan and other dye-labeled primers or probes please refer to Bio-Rad Laboratories' Real-Time PCR Applications Guide (1709799EDU).

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. This is similar to the action of ethidium bromide, but unlike 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 double-stranded DNA, and is not hazardous.

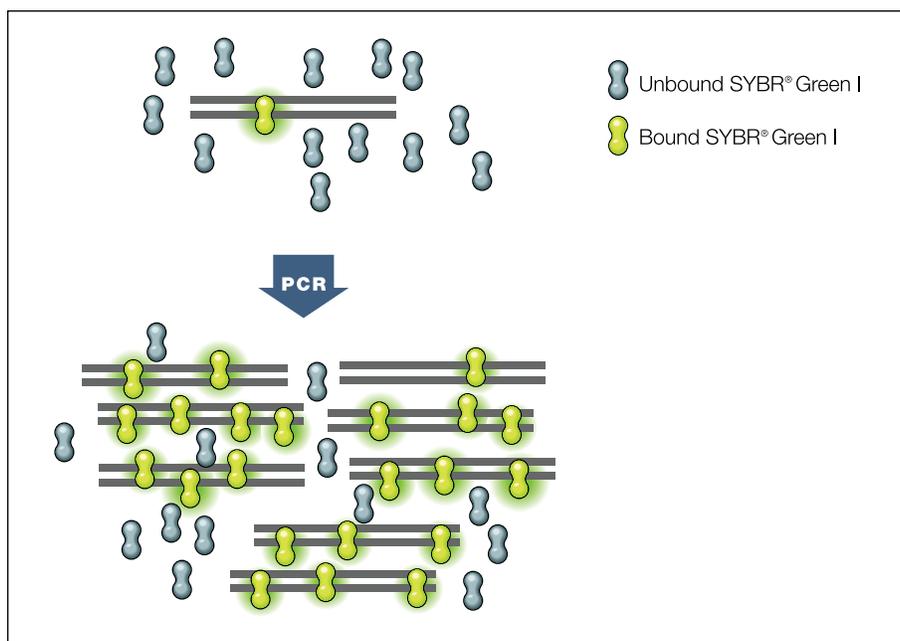


Figure 3. DNA-binding dyes in real-time PCR. Fluorescence dramatically increases when the dye molecules bind to double-stranded DNA.

Other advantages of using a dye that binds only to dsDNA such as SYBR® Green I include simple assay design (only two primers are needed; probe design is not necessary) ability to test multiple genes quickly without designing multiple probes (for example, 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.

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The main disadvantage to the use of SYBR® Green I is its nonspecificity. Since it will bind to any double-stranded DNA, you cannot distinguish between the amplification of target DNA and the amplification of primer-dimers. Also, if non-target sequences are being amplified, this will show up 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; this is commonly done 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. 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 as the dsDNA becomes denatured. 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 vs. temperature ($-d(\text{RFU})/dT$). 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.

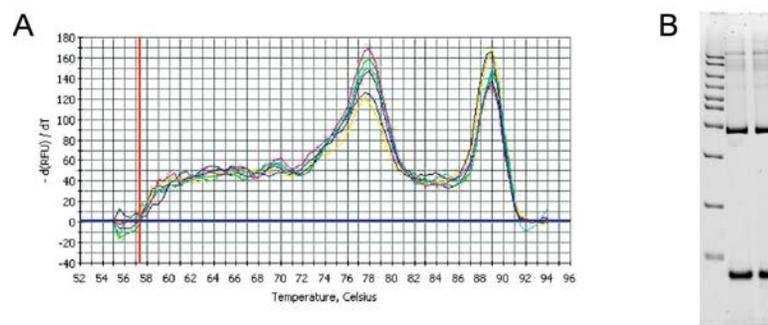


Figure 4. Melt-curve analysis of reaction product from a SYBR® Green I assay. The melt-curve analysis function of real-time instruments can be used to distinguish specific products from non-specific products. **A.** The negative first derivative of the change in fluorescence is plotted as a function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

Experimental Protocol

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.

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 electrophoresis.

Laboratory 2 is more advanced, and modifies the Crime Scene Investigator kit protocol to include a DNA dilution series and an optional very fast PCR protocol. This lab allows for a rapid demonstration of the quantitative nature of real-time PCR and melt-curve analysis, and in doing so uses an entirely different experimental design than the core Crime Scene Investigator kit.

Educators not familiar with the basic principles of real-time PCR may also wish to purchase the Bio-Rad Real-Time PCR Applications Guide (1709799EDU), as it provides an excellent tutorial on the theory and application of quantitative PCR. Bio-Rad's Amplification Central is also an excellent online source of information on real-time PCR including PCR Doctor, animations and other interactive tools. For more information please visit us at www.bio-rad.com/genomics/pcrsupport

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

Introductory Level

Purpose

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

Laboratory 1 involves running the Crime Scene Investigator PCR kit on a real-time PCR instrument, and substituting a master mix with fluorescent dye (iQ™ 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)
- iQ™ SYBR® Green Supermix (1708880EDU)
- PCR-grade water (proteomics-grade water is also acceptable, 1632091EDU)

Additional required items

- PCR tube strips (TLS0851EDU for the CFX Connect™)
- Optical flat caps (TCS0803EDU for the CFX Connect)
- A real-time PCR instrument such the CFX Connect (1855200EDU)
- 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 iQ™ SYBR® Green supermix for the PCR mastermix 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 the CFX Connect™ call 1-800-4BIO-RAD in the US 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 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	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 (such as the CFX Connect or the iQ5), 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 real-time thermal cycler with the following 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 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.

A Real-Time PCR Extension

Please note Laboratory 2 has an alternative cycling protocol designed to obtain results in about 1 hour (see page 12 for Lab 2). However, this fast protocol will preferentially amplify the smaller suspect target DNA allele sequences (200 and 300 bp) and will not allow students to match the crime scene with a suspect on an agarose gel at the end of the activity since not all bands will be amplified.

Laboratory 1 Expected Results

The graph below shows what the fluorescence of the PCR reactions looks like, observed in real time as the Crime Scene Investigator PCR reactions amplified. Total fluorescence is on the y-axis of the graph, and the PCR cycle number is on the x-axis.

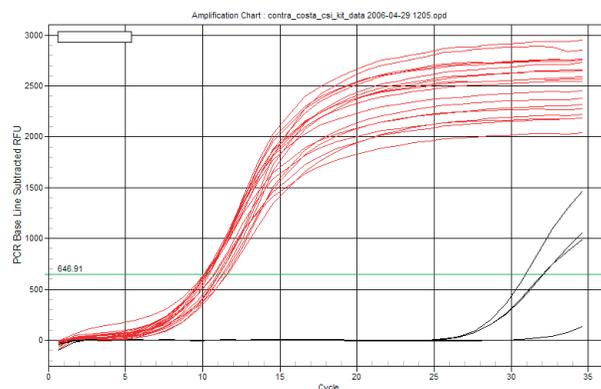


Figure 6. PCR Amplification plot. Color key: red = CSI kit reactions, black = no template control.

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 and that no more amplification was going on. Interestingly, samples without any template DNA (the “no template control”) began to show amplification very late in the PCR cycling (after cycle 30). These amplification products were PCR primer-dimers, resulting from 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 the DNA is being 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 resulting in a decrease in fluorescence. Different amplicons will dissociate at different temperatures based upon the length of the amplicon and the GC content. Melt-curve analysis for this data set will 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.

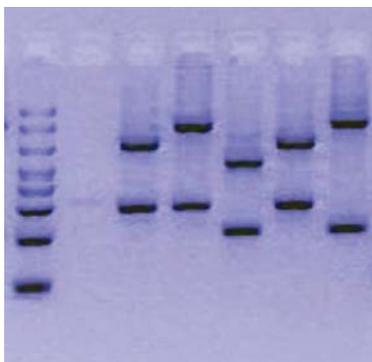


Figure 7. Real-time PCR products separated on a 3% agarose gel. Lanes from left to right: allele ladder, NTC, CS, Suspects A, B, C, and D.

In Figure 7, the crime scene DNA (lane CS) clearly matches the band pattern for the DNA obtained from suspect C, and clearly differs from the DNA obtained from the other three suspects. No visible band is present in our no template control (NTC) lane. 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 PCR by evaluating a dilution series, of known DNA, with real-time PCR reagents and instrumentation.

Materials

- Crime Scene Investigator PCR Basics Kit (1662600EDU)
- Small DNA Electrophoresis Reagent Pack (1660450EDU)
- iQ™ SYBR® Green Supermix (1708880EDU)
- PCR-grade water (proteomics-grade water is also acceptable, 1632091EDU)

Additional Required Items for qPCR

- PCR tube strips (such as TLS0851EDU for the CFX Connect™)
- Optical flat caps (such as TCS0803EDU for the CFX Connect)
- A real-time PCR instrument such as the CFX Connect (CFB3120EDU)
- 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.

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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 Connect™ 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 the CFX Connect or the iQ5™), 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 & Extension- collect data

Repeat Cycle 2 for 40 cycles

A Real-Time PCR Extension

Student Protocol

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 will allow you to obtain results for the 200 and 300 bp alleles in around 1 hour. However, this protocol does not amplify the higher molecular weight alleles (500, 700 and 1000 bp). If desired, use the protocol in Lab 1 (page 10) which will amplify all the alleles.

Thaw Tubes

1. On ice, thaw the DNA samples and primers from the Crime Scene Investigator kit and the iQ™ 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 Mastermix

6. Label a fresh 1.5 ml microtube "MM".
7. Pipet 110 µl of iQ™ 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.

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Add Mastermix to PCR Tubes

9. Add 12.5 µl of mastermix 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 15. Those diluted 1/10,000 fold will appear later, perhaps around cycle 25. Finally, those samples diluted 1/1,000,000 will appear last, after cycle number 30. The no template control will contain PCR primers and will also begin to amplify around cycle 30.

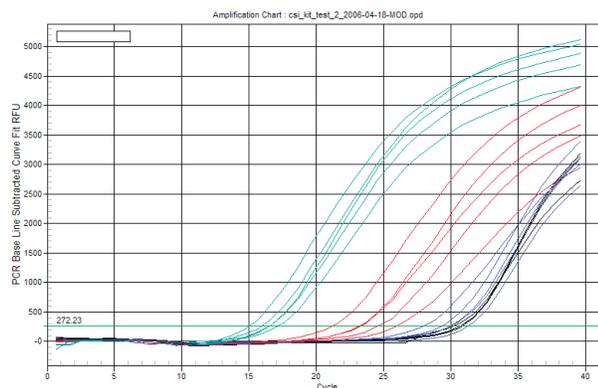


Figure 9. PCR Amplification plot. Color key: Green=100X, Red=10,000X, Blue=1,000,000X, Black=NTC.

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 threshold cycle (commonly called the C_T value) directly relates to the starting quantity of template. These C_T 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_T values that differ by 3 cycles would represent $2^3 = 8$ -fold difference in starting template concentration.

A Real-Time PCR Extension

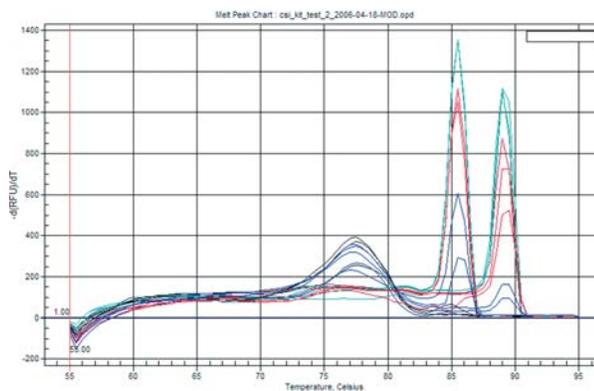


Figure 10. Melt-curve plot. Color key: Green=100X, Red=10,000X, Blue=1,000,000X, Black=NTC.

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 individual reaction will have two peaks: one representing the 200 bp amplicon (suspects B and D) or one representing the 300 bp amplicon (suspects A and C), and a 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, while the two peaks above 85°C represent the 200 and 300 bp amplicons from different suspect DNA 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

Since Bio-Rad's real-time PCR reagents (the iQ™ SYBR® Green supermix) can directly replace the PCR Mastermix 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) take a very long number of 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 clearly 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 $E = 10^{-1/\text{slope}}$. The percent amplification efficiency is calculated using the formula $\%E = (E - 1) \times 100\%$, 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	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_T	See threshold 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 molecule.

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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 mRNA for subsequent translation into protein in a living organism. 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 thought to be unaffected 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".

A Real-Time PCR Extension

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.
qPCR	Quantitative PCR. Also called real-time PCR. A technique that uses fluorescently labeled molecules to track the accumulation of amplified products with each cycle of PCR.
r	See correlation coefficient.
R²	See coefficient of determination.
Real-time PCR	Also called quantitative PCR or qPCR, 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 _T 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.

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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.
Threshold	In real-time PCR, the level of fluorescence that is considered to be significantly above the baseline level measured in the early cycles. A valid threshold value may be automatically selected by software-based algorithms, or may be manually selected during real-time PCR data analysis.
Threshold cycle (C_T)	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 C _T value is used to accurately and reliably measure the initial amount of template present in the reaction.
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.

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