Application Note

Biotechnology Explorer GMO Investigator Kit:
A Quantitative Real-Time PCR Extension

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Introduction

This application note covers the use of the GMO Investigator Kit as a foundation for a teaching lab exercise and use of real-time PCR technology.

The Biotechnology Explorer GMO Investigator kit is a tool for teaching students the principles of the polymerase chain reaction (PCR) and its use in testing foods for genetic modifications. Real-time PCR is an extremely important technology, useful not only in food analysis, but also in gene expression analysis and many other applications in which the goal is not only to ask "what DNA is present" but also "how much".

The Bio-Rad GMO Investigator kit is a popular tool for demonstrating PCR in the classroom. Using this kit, students can extract DNA from various food samples and then examine the DNA by PCR to determine whether the food has been genetically modified. To teach the basics of real-time PCR in the classroom with the GMO Investigator kit, 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 Bio-Rad PCR instrument such as the CFX Opus or CFX Duet Real-Time PCR System. Although the GMO Investigator 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. Using this extension with the GMO Investigator kit can show how much plant DNA, and compare how much genetically modified organism (GMO) DNA, is recovered from each food sample. It is even possible to determine what fraction of a food product has been made with genetically modified ingredients, in almost exactly the same manner as standard testing labs do.

Learning Objectives

In this exercise, students will:

- Use the GMO Investigator kit to prepare and analyze authentic food samples for both plant DNA and GM DNA content
- 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

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. In food testing labs, real-time PCR is used to test for food integrity, food contamination, and GMO content of food.

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 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.

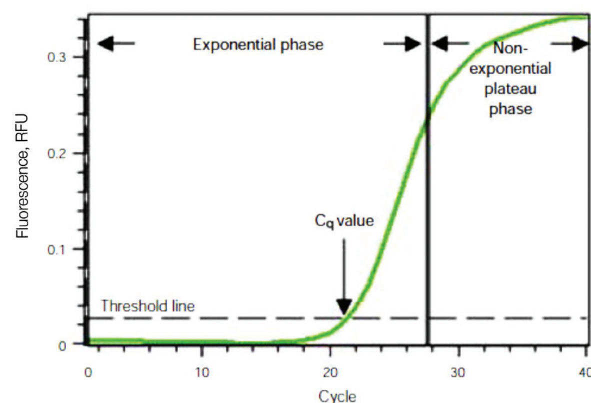


Figure 1. Amplification plot. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds and reaction components are consumed, the reaction slows and enters the plateau phase.

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 quantification cycle, or C_q. 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.

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 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_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.

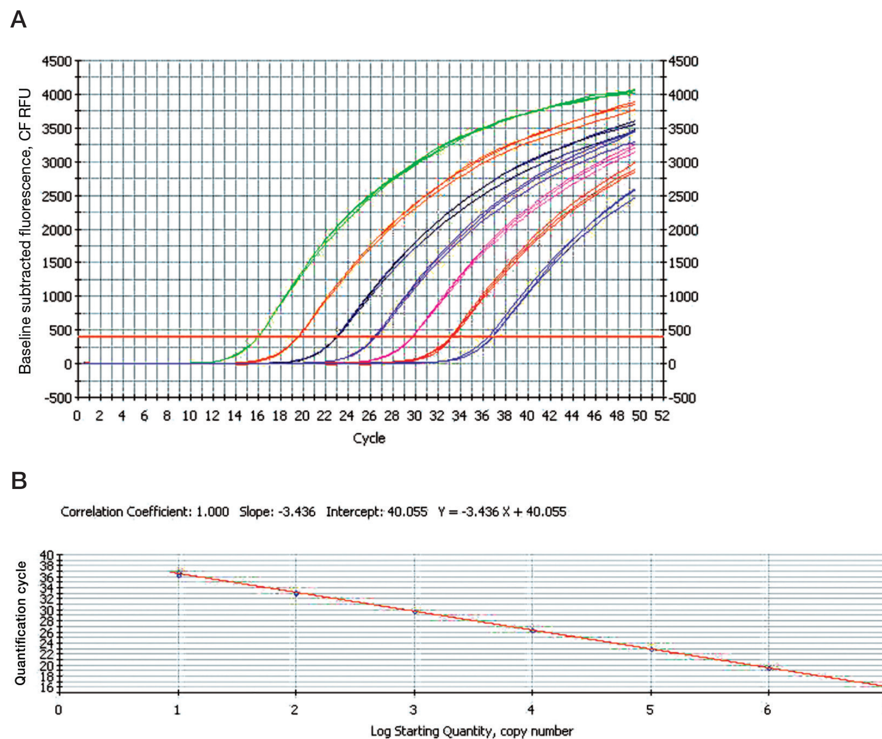


Figure 2. Generating a standard curve to assess reaction optimization. A standard curve was generated using a 10-fold dilution of a template amplified on the iCycler iQ Real-Time System. Each dilution was assayed in triplicate. **A.** Amplification curves of the dilution series. **B.** Standard curve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

The 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_q values between replicates will lower the r or R^2 value. You should strive for an r whose absolute value is >0.990 or an R^2 value >0.950 for your qPCR reactions.

Singleplex or Multiplex Reactions for Real-Time PCR

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

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

If none of these considerations are particularly important for your assays, then singleplex reactions are sufficient.

The GMO Investigator kit uses multiplex PCR in the GMO reactions, while the plant primer reactions are singleplex. Please note that the “multiplex” in the GMO Kit is slightly different than the “multiplexing” described above. In the GMO Investigator kit, primer pairs are mixed (multiplexed) together but are detected simultaneously, either as bands on an agarose gel or as a SYBR® Green I dye signal in this real-time extension. In the description above, primer pairs also include a matched fluorescent probe for each primer set, which allows multiple PCR reactions to be detected independently in the same vial (a different form of “multiplexing”). Since this real-time extension uses a single-color SYBR® Green I reaction, even though we are using multiplexed primers, we will not use multiplexed detection. The two pairs of GMO primers in the kit will amplify two DNA sequences, a 203 base pair (bp) fragment of the cauliflower mosaic virus (CaMV) 35S promoter and a 225 bp fragment of the nopaline synthase (NOS) terminator. The kit is designed to use multiplex PCR with the GMO reactions so that a greater range of genetically modified (GM) foods can be detected, since some foods contain just the CaMV 35S promoter, some just the NOS terminator, and some foods contain both. By testing for both sequences in a single reaction, approximately 15% more GM foods can be detected than if only the CaMV 35S primers were used. Please note that foods with compound genetic modifications, such as corn, with both round-up ready resistance and B_t modification, may also have multiple copies of these regulatory sequences. Multiplexing in this case also reduces reagent costs and minimizes sample handling, which is particularly important during this activity since contamination can be quite common if extreme care is not taken. For more information regarding multiplexing and GMO detection, please refer to Bio-Rad’s Real-Time PCR Applications Guide, Rev B (bulletin 5279).

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's Real-Time PCR Applications Guide, Rev B (bulletin 5279).

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.

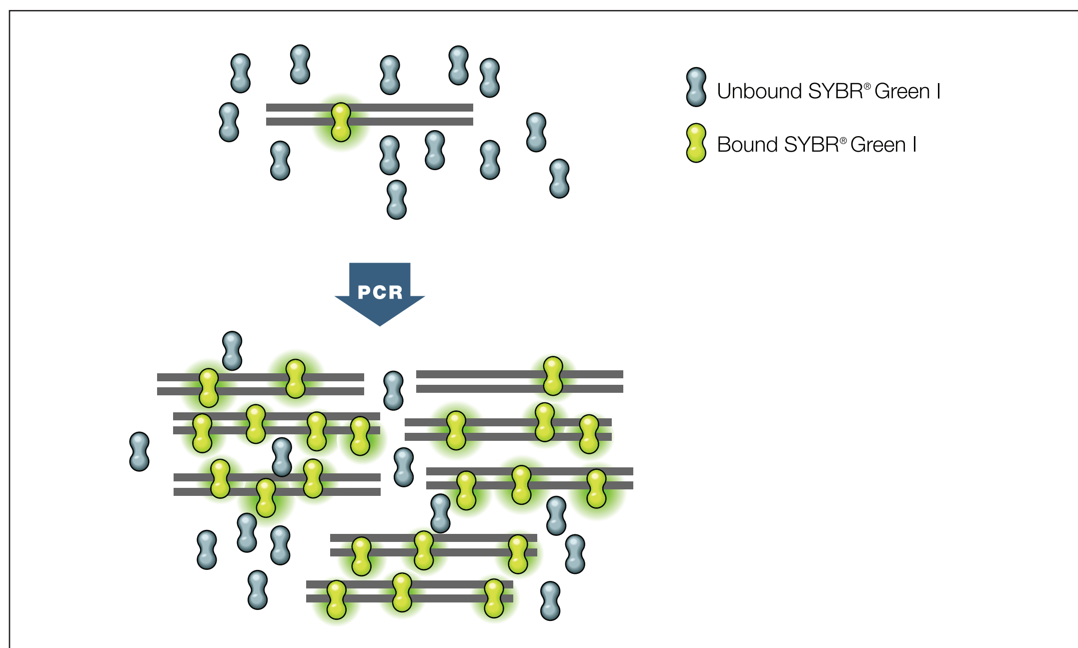


Figure 3. DNA-binding dyes in real-time PCR. Fluorescence dramatically increases when the dye molecules bind to double-stranded DNA.

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.

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(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 Tm of 78°C represents the nonspecific product, and corresponds to the lower bands in lanes 2 and 3 on the gel.

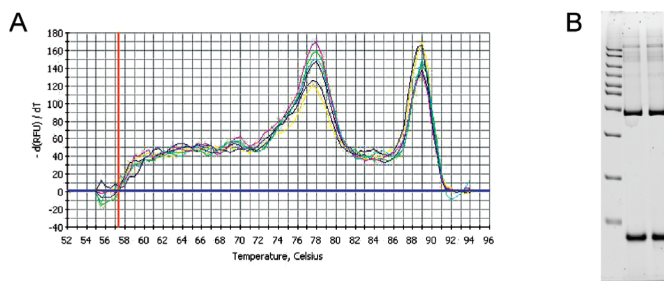


Figure 4. Melt-curve analysis of reaction product from a SYBR® Green I assay. The melt-curve analysis function of real-time instruments can be used to distinguish specific products from non-specific products. **A.** The negative first derivative of the change in fluorescence is plotted as a function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

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 where possible, such as 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 and to ensure the reactions are at the bottom of the tubes.

Experimental Protocol

Overview

The GMO Investigator kit is designed to test for the presence of two different GMO-associated DNA sequences: the 35S promoter of the cauliflower mosaic virus, and the terminator of the nopaline synthase gene of *Agrobacterium tumefaciens.* These DNA sequences are present in most of the GM crops that are approved for distribution worldwide. In addition, the integrity of the plant DNA extracted from food is tested by amplifying a section of the photosystem II (PSII) chloroplast gene that is common to most higher plants. The results are visualized on an agarose gel. For more information, see the GMO Investigator kit instruction manual. 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 allows you to carry out a simple DNA dilution series — prior to carrying out complex experiments with real-time PCR. By doing this simple experiment with the reagents and instruments that you will be using in later experiments, you will have a much better idea of what to expect from the more complex real-time analysis of actual food samples. In addition, the dilution series will not only demonstrate the quantitative nature of real-time PCR, it will also verify that your reagents and instrument are working properly. Laboratory 1 allows students to observe real-time PCR as the reactions progress and allows the students to amplify the GMO positive control with the GMO primers as well as with the plant PSII primers. Students can then compare the results of samples using agarose gel electrophoresis.

Laboratory 2 is more advanced, and modifies the GMO Investigator kit protocol. We recommend performing Laboratory 1 first so that you will be better versed in real-time PCR and equipped with the tools and knowledge necessary for evaluating the DNA content of actual food samples via real-time PCR. The principles to follow are exactly the same as outlined in Laboratory 1, with the exception that rather than using a known dilution series of DNA, you will instead use the DNA extracted from food samples using the extraction protocol in the GMO Investigator kit. This lab allows for a demonstration of the quantitative nature of real-time PCR as well as melt-curve analysis.

For more information to learn more abot Bio-Rad's advanced PCR products, please visit https://www.bio-rad.com/en-us/category/genomics?ID=2d11dcf8-2dbe-47a5-a1de-8315abd3c17e

Laboratory 1: GMO Investigator Kit Protocol, Run on a Real-Time PCR Instrument

Introductory Level

Purpose

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

Laboratory 1 involves running the GMO Investigator PCR kit on a real-time PCR instrument, and substituting a master mix with fluorescent dye (SsoAdvanced Universal SYBR® Green Supermix) for the mastermix 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

- GMO Investigator Kit (1662500EDU)
- Small Fast Blast DNA Electrophoresis Reagents Pack (1660450EDU)
- SsoAdvanced Universal SYBR® Green Supermix (1725270EDU)
- PCR-grade water (proteomics-grade water is also acceptable, 1632091EDU)

Additional required items

- PCR tube strips (TLS0851EDU)
- Optical flat caps (TCS0803EDU)
- A real-time PCR instrument such as the CFX Opus or CFX Duet Real-Time PCR System
- Agarose gel electrophoresis equipment
- Equipment as described in the GMO Investigator Kit manual

Instructor's Overview

- 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 with different master mixes: plant master mix (PMM) or GMO master mix (GMM) or by performing replicate reactions
- 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
- Substitute the master mix from the GMO Investigator Kit with SsoAdvanced Universal SYBR® Green Supermix
- 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 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 Systems call 1(800)4-BIO-RAD in the US or contact your local Bio-Rad office.

Plate Setup

See Figure 5 for a suggestion of 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.

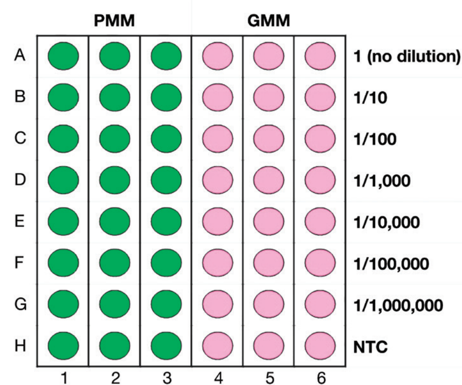


Figure 5. Plate setup. Columns 1–3 represent samples with plant master mix (PMM). Columns 4–6 represent samples with GMO master mix (GMM). Rows A–G represent the addition of GMO-positive control DNA at the noted dilutions. Row H represents the no template control (NTC).

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 Opus or CFX Duet Real-Time PCR 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 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:	95°C for 4 min	Initial denaturation of DNA
Cycle 2:	94°C for 1 min	Denaturation
	59°C for 1 min	Annealing
	72°C for 2 min	Extension — collect data after this step
	Repeat Cycle 2 for 40 cycles	

Cycle 3 (Optional): 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 sec 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.

Preparing the Reagents

1. Locate the following reagents:
   - SsoAdvanced Universal SYBR® Green Supermix
   - GMO-positive control DNA
   - Plant primers
   - GMO primers

2. Thaw all reagents on ice and mix by vortexing or gently tapping the tubes.

3. Spin down in centrifuge to force solutions to the bottom of the tubes (10 sec at full speed).

4. Keep on ice.

Preparing the DNA samples

5. Locate the GMO-positive control DNA in the GMO Investigator kit.

6. Label eight screwcap tubes #1–8 for the following standard dilutions: no dilution (1), 1/10, 1/100, 1/1,000, 1/10,000, 1/100,000, 1/1,000,000, and no template control (NTC).

7. Add 50 µl of GMO-positive control DNA to the tube labeled #1 for the no dilution sample.

8. Add 90 µl of sterile distilled water to each of the tubes labeled 2-8.

9. Add 10 µl of GMO-positive control DNA to the tube labeled #2 for the 1/10 dilution. Pipet up and down repeatedly to make sure all DNA is rinsed from the tip and mixed thoroughly. Mix the tube thoroughly by vortexing for at least 10 sec or by flicking the tube at least 20 times.

10. Perform serial dilution of DNA:
    Transfer 10 µl diluted DNA from tube #2 to tube #3. Mix thoroughly.
    Transfer 10 µl diluted DNA from tube #3 to tube #4. Mix thoroughly.
    Transfer 10 µl diluted DNA from tube #4 to tube #5. Mix thoroughly.
    Transfer 10 µl diluted DNA from tube #5 to tube #6. Mix thoroughly.
    Transfer 10 µl diluted DNA from tube #6 to tube #7. Mix thoroughly.
    Tube #8 will have no DNA template added for the "no template control" (NTC).

    In each case, pipet up and down repeatedly to make sure all DNA is rinsed from the tip and mixed thoroughly. Mix the tube thoroughly by vortexing for at least 10 sec or by flicking the tube at least 20 times.

Preparing the PCR Tubes

11. For each dilution series obtain an eight well PCR tube strip. Label each tube 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 analyzing the plant primers with the GMO Positive Control DNA would have the tube labeled as follows: PMM 1, PMM 1/10, PMM 1/100, PMM 1/1000, PMM 1/10,000, PMM 1/100,000, PM 1/1,000,000, and PMM NTC. Ensure that the reactions are set up in the exact manner programmed into the real-time PCR instrument.

Preparing the Master Mix

12. For each dilution series of 8 tubes you will need 150 µl of 2x soAdvanced Universal SYBR® Green Supermix and 1.5 µl of either green PSII plant or red GMO primers depending on the experimental plan determined by the instructor. If more than one dilution series will be performed, increase the volumes appropriately. Carry out all of the following steps on ice.

13. Label two screwcap tubes as follows:
    - "PMM" for "plant master mix"
    - "GMM" for "GMO master mix"

14. Add 150 µl of the SsoAdvanced Universal SYBR® Green Supermix to both the PMM and GMM tubes.

15. Add 1.5 µl of plant primers to the tube labeled "PMM". Pipet up and down repeatedly to make sure all DNA is rinsed from the tip; mix thoroughly, spin down.

16. Add 1.5 µl of GMO primers to the tube labeled "GMM". Pipet up and down repeatedly to make sure all DNA is rinsed from the tip; mix thoroughly, spin down.

Adding Master mix to PCR Tubes

17. Aliquot 12.5 µl of plant master mix (PMM) to each of the 8 PCR tubes in the labeled 8-tube strip. Make replicates if dictated by the experimental plan provided by the instructor.

18. Aliquot 12.5 µl of GMO master mix (GMM) to each of the 8 PCR tubes in a second labeled 8-tube strip. Make replicates if dictated by the experimental plan provided by the instructor.

19. The strip of completed tubes should look similar to the following:

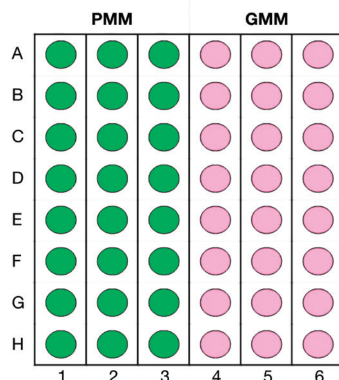


Figure 6. Master mix plate setup. Columns 1–3 represent samples with plant master mix (PMM). Columns 4–6 represent samples with GMO master mix (GMM).

Adding the DNA Samples to the Tubes

20. Add 12.5 µl of tube #1 (1 — no dilution) to the appropriate reaction(s) in the PCR tube strip. Be sure to use a fresh pipet tip each time. Mix each 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 mixture.

21. Repeat the above step for the remaining DNA dilutions (tubes #2 through #7) and for the NTC control reactions as well.

22. The completed dilution series should look similar to the following:

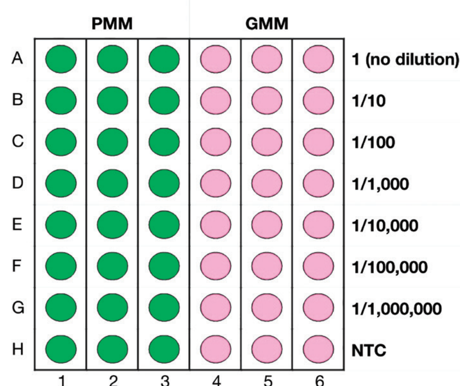


Figure 7. Plate setup. Columns 1–3 represent samples with plant master mix (PMM). Columns 4–6 represent samples with GMO master mix (GMM). Rows A–G represent the addition of GMO-positive control DNA at the noted dilutions. Row H represents the no template control (NTC).

Running PCR Reactions

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

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

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

Creating Standard Curves (Optional)

26. Most real-time instruments contain the software to create standard curves. Please refer to the instrument's instruction manual for details on constructing a standard curve. Alternately, it is possible to download the data into a graphing computer program and create a standard curve or students can draw a standard curve on semilog graph paper. A standard curve can be generated with a serial dilution of template DNA. An inverse correlation exists between the log of the quantity of DNA (x-axis) and the C_q value (y-axis). PCR efficiency can be calculated from the slope of the standard curve. The assay efficiency should range from 90–110%. You should have two standard curves: one for reactions using plant primers (PMM) and one for reactions using GMO primers (GMM).

Gel Electrophoresis Analysis (Optional)

Remember that real-time PCR gives specific information about the quantity of starting template, whereas agarose gel electrophoresis gives specific information about the size of the amplicons produced. The plant primers in the GMO kit will produce an amplicon of approximately 455 bp, and the GMO primers will produce 1–2 amplicons of approximately 203–225 bp. However, in the absence of starting template (for example, in the NTC or the 1/1,000,000 dilution), those same primers will produce a much smaller primer-dimer amplicon.

Real-time PCR plots for the true PCR product and for the artifact primer-dimers look very similar, yet the band pattern for these two different amplicons looks dramatically different on an agarose gel. Therefore, running an agarose gel of the PCR products in the dilution series is highly recommended — so that the user may gain experience in interpreting real-time results that may contain both true product (plant or GM amplicons) and artifacts (primer-dimers).

27. Prepare 3% agarose gels as described in the GMO Investigator kit protocol, with enough lanes for the analysis of at least one set of dilutions (including NTC) from the plant primers and one set from the GMO primers plus a lane for the PCR molecular weight ruler (this would require 17 lanes).

28. Add 6 µl of Orange G loading dye (provided in the GMO Investigator Kit) to each 25 µl PCR reaction that will be loaded on the gel.

29. Add 50 µl of Orange G loading dye to the vial of PCR molecular weight ruler. Mix well and pulse-spin.

30. Load 20 µl of each of the PCR samples, as well as 20 µl of the PCR molecular weight ruler on the agarose gel.

31. Run the agarose gel at 100 V for 30 min.

32. Analyze the results as described in the GMO Investigator Kit protocol. Can you determine starting DNA concentrations from the gel data?

Laboratory 1 Expected Results

The protocol described in this section will create a DNA template dilution series. The realtime PCR reactions will show up as curves similar to those shown in the following graph. The 1 (no dilution) DNA, being most concentrated, will have curves that appear "earliest", for example, before cycle 10. Those diluted more will appear later (or further to the right). Since the position of the amplification curves on a real-time PCR instrument are entirely dependant and proportional to the starting concentration of DNA, as the DNA becomes more dilute, the curves will be shifted more and more to the right. An example plot from a dilution series is shown on the next page.

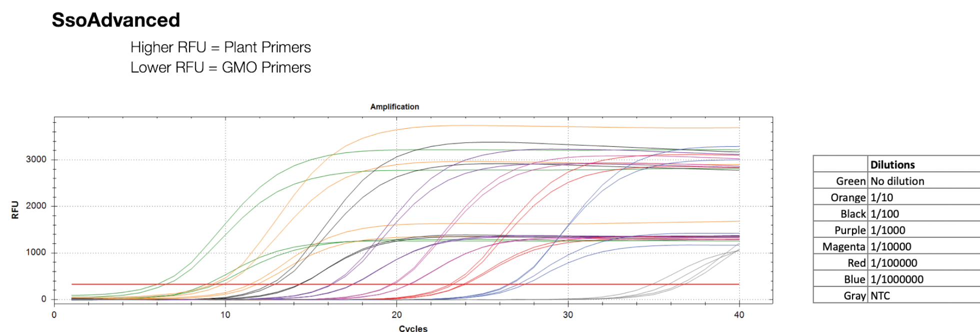


Figure 8. PCR amplification plot. Color key: green = 1x, orange = 10x, black = 100x, purple = 1000x, magenta = 10,000x, red = 100,000x, and blue = 1,000,000x, NTC = gray. Note that fluorescence units are shown in relative fluorescent units (RFU). Line graphs at higher RFUs correspond to plant primers while the lower line plots reflect GMO primer results.

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 quantitation 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 three cycles would represent 2^3 = an 8-fold difference in starting template concentration.

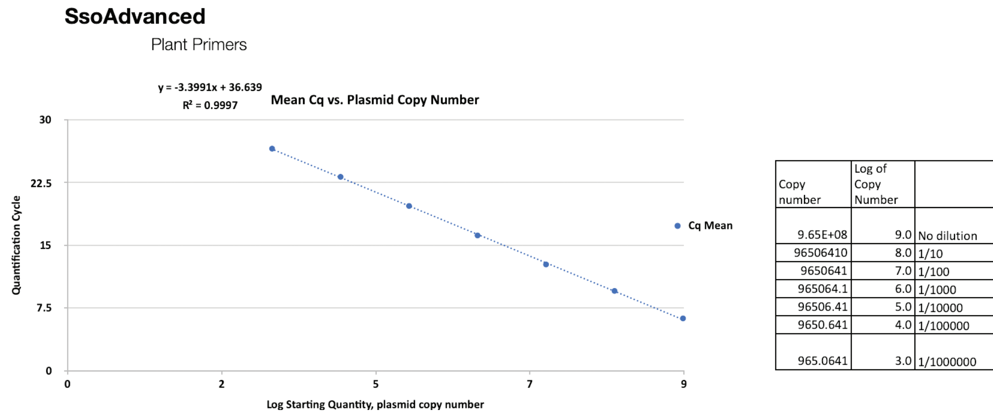
Please consult the Bio-Rad Real-Time PCR Applications Guide, Rev B (bulletin 5279). if you are new to real-time PCR and need background information on interpreting real-time PCR results. Interpretation of real-time data can be complicated and difficult. Bio-Rad Laboratories' technical support team is an excellent resource to help answer questions about real-time PCR and data interpretation.

Consistency of replicates. Since duplicate sets of each PCR reaction were prepared, do the replicates have similar results? If they don't, what could be some possible reasons for the discrepancy? Perhaps pipeting error or bubbles in the PCR reaction have resulted in differences between replicates.

Differences between the results from the plant primers and the GMO primers.
Are there any significant differences between the curves for the two separate sets of primers? Typically, the GMO amplification products fluoresce less brightly than the plant amplification products. Since the fluorescent dye used in the real-time PCR reaction is SYBR® Green I (which happens to fluoresce at a green wavelength), the red dye in the GMO primers solution in the GMO Investigator kit quenches some of the green fluorescence signal from these reactions. The small amount of red dye slightly reduces the SYBR® Green I fluorescence, but does not eliminate it.

Spacing of the dilution curves. Which dilutions were the first to cross the threshold line and which were the last? Are the dilutions more-or-less evenly spaced? When a PCR reaction is running at 100% efficiency, in other words a perfect doubling of product every cycle, then it will take approximately 3.3 cycles to multiply the product 10-fold ($2^{3.3}\approx10$). Therefore, the 10-fold dilution series in this experiment should have the curves spaced around 3.3 cycles apart.

Analyzing standard curves (Figure 9). Are all of the points on a straight line? The correlation coefficient (r^2 value) for the best-fitting straight line is an indicator of the straightness of the line. An r^2 of 1.00 is a perfect line, and an r^2 value greater than 0.98 is generally acceptable in quantitative real-time PCR standard curves. Imagine using the standard curve to calculate “unknown” DNA quantities from a known C_q value. If you were quantitating unknown DNA template, could you trust the quantities calculated from curves that are almost identical to your no-template control?



Copy number	Log of Copy Number	
9.65E+08	9.0	No dilution
96506410	8.0	1/10
9650641	7.0	1/100
965064.1	6.0	1/1000
96506.41	5.0	1/10000
9650.641	4.0	1/100000
965.0641	3.0	1/1000000

Figure 9. Standard curve. C_q values for the standards are on the x-axis, and the corresponding quantity of starting template DNA for each standard is on the y-axis. A well-optimized assay will have a standard curve similar to this, in which the points all fall close on a straight line. The spacing of the points is controlled by the dilution series used.

Amplification in the NTC (no template control). Did you see any amplification in the no-template controls? Since there was no template in these reactions, why was there amplification? In the absence of sufficient quantities of template, the DNA polymerase in the PCR reaction will actually amplify any primers that have spontaneously annealed to each other, creating primer-dimers. These will be further amplified as PCR continues. Notice that, by looking at the graphs, there is no easy way to tell the difference between the true PCR products and the primer-dimers. How would you determine whether your real-time PCR amplification reactions are actually creating primer-dimers?

Melt-curve analysis (Figures 10A and B). Did you run a melt-curve of your PCR amplicons? If so, is there a noticeable difference in the melting temperature profiles between the plant amplicons and the GMO amplicons? Since the plant amplicons are almost double the length of the GMO amplicons (455 vs. 203–255 bp), they will have a slightly higher melting temperature peak. Were there detectable primer-dimers in the no template control? If so, notice how they have a much lower melting temperature peak, since those amplicons are very short. Use the melt-curve analysis to get an idea of which amplicons were produced in each of your PCR samples.

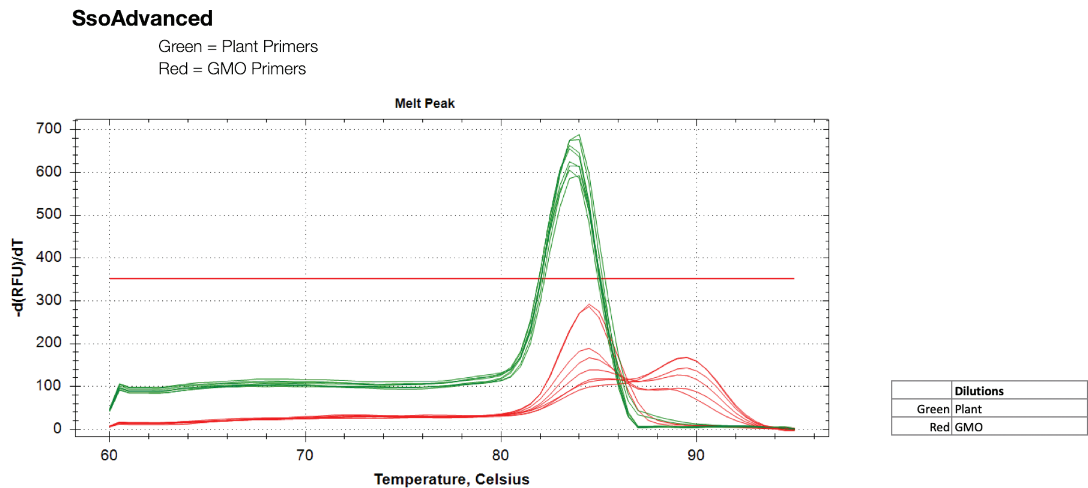


Figure 10A. Melt-curve plot. Color key: green = plant primers and red = GMO primers. 1x and NTC dilutions are not shown on this plot. The main peak represents the melting temperature of the amplicons in this reaction. Off-target amplification is not apparent in the plant primer samples while multiple amplicons are apparent in GMO amplification samples.

Agarose gel electrophoresis. How did the agarose gels look? Did all of the plant PCR reactions have a 455 bp band and all of the GMO reactions have 203–255 bp band(s)? Is there any evidence of primer-dimers in the no template control? How about evidence of primer-dimers in the most dilute template sample (1/1,000,000)? Note how the quantitative real-time PCR and the agarose gels provide very different, but equally valuable, pieces of information.

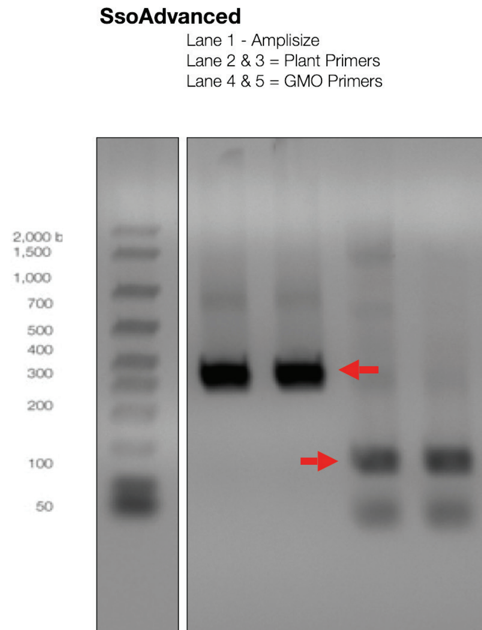


Figure 10B. Melt-curve plot. A. Color key: green = plant primers and red = GMO primers. 1x and NTC dilutions are not shown on this plot. The main peak represents the melting temperature of the amplicons in this reaction. Off-target amplification is not apparent in the plant primer samples while multiple amplicons are apparent in GMO amplification samples. **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 plant primer reaction shown in (A); lanes 4 and 5, two replicates of qPCR product from the GMO primer reaction shown in (A). The two correct PCR products are revealed by separate bands in the gel (black arrows). Less prominent bands are also apparent, particularly primer dimer bands in GMO (asterisk). These amplicons can give additional melt curve peaks.

Laboratory 2: Evaluation of GMO Foods

Advanced Level

Purpose

After the completion of Laboratory 1, your students will be equipped with the tools and knowledge necessary for evaluating the DNA content of actual food samples. The principles to follow are exactly the same as outlined above, with the exception that rather than using a known dilution series of DNA, the DNA extracted from food samples will be used.

For the majority of this laboratory, the directions in the GMO Investigator kit should be followed as the procedures involved are essentially the same, with the exception that the reagents and experimental design are slightly different as indicated in the following instructions.

Materials

- GMO Investigator Kit (1662500EDU)
- Small Fast Blast DNA Electrophoresis Reagents Pack (1660450EDU)
- SsoAdvanced Universal SYBR® Green Supermix (1725270EDU)
- PCR-grade water (proteomics-grade water is also acceptable, 1632091EDU)

Additional Required Items

- Test food(s) from grocery store
- PCR tube strips (TLS0851EDU)
- Optical flat caps (TCS0803EDU)
- 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 GMO Investigator Kit manual

Instructor's Overview

- This laboratory culminates in students finding the relative level of GMO content of one food compared to another after the GMO results are normalized against a reference chloroplast gene. To ensure that the normalization is valid we highly recommend using foods from a common food source; for example, comparing corn-based foods to each other or comparing soy-based foods to each other, but not comparing corn- to soy-based foods. In addition, since comparing levels of GMO content requires that both foods are GMO positive it would be advisable for instructors and/or the class to test potential foods using the conventional GMO Investigator kit protocol prior to the real-time PCR laboratory to confirm that the foods being tested are GMO positive
- 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
- 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

Experimental Design and Instructor Preparation

Planning the experiment. Depending on your teaching goals and the level of your students, you may choose to plan the experiment yourself and have students perform the lab based upon your experimental design or you may choose for them to plan their own experiment in addition to performing the lab.

The negative control should be PCR-grade water or blank InstaGene supernatant. The positive control should be the GMO-positive control plasmid. The primers should be used at half the concentration stated in the GMO Investigator manual to reduce quenching of the fluorescence signal — such that every 100 µl of 2x supermix should have 1 µl of primers added.

The following should be considered to help determine the experimental design:

- Which food samples will be analyzed for GMO content
- What the negative control will be
- What the positive control will be
- How many replicate samples to include

Remember, each DNA template will be tested with two different primer sets; universal plant PSII primers test for the presence of amplifiable plant DNA and GMO primers test for the presence of a genetic modification in the plant's genome.

Determine the plate setup for the experiment. It is very important to ensure that the PCR reactions are the same as what is specified in the Plate Set Up file.

An example plate setup is shown below:

	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	NTC PMM	NTC PMM	NTC PMM	NTC PMM	NTC PMM	NTC PMM
B	Food 1 PMM	Food 1 PMM	Food 1 PMM	Food 3 PMM	Food 3 PMM	Food 3 PMM
C	Food 2 PMM	Food 2 PMM	Food 2 PMM	Food 4 PMM	Food 4 PMM	Food 4 PMM
D	POS PMM	POS PMM	POS PMM	POS PMM	POS PMM	POS PMM
E	NEG GMM	NEG GMM	NEG GMM	NEG GMM	NEG GMM	NEG GMM
F	Food 1 GMM	Food 1 GMM	Food 1 GMM	Food 3 GMM	Food 3 GMM	Food 3 GMM
G	Food 2 GMM	Food 2 GMM	Food 2 GMM	Food 4 GMM	Food 4 GMM	Food 4 GMM
H	POS GMM	POS GMM	POS GMM	POS GMM	POS GMM	POS GMM

Figure 11. Sample experimental plate setup. Each column A–H represents an 8 well strip. "NTC" refers to "no template control". PMM refers to master mix with plant primers and GMM refers to master mix with GMO primers. "POS" refers to a "positive control".

Construct a table with the headings: Strip Number, Tube Letter, Template and Primers. Complete the table, ensuring each PCR reaction that is to be run is planned for andensuring the reactions align with the plate setup programmed into the real-time PCR instrument.

Identify and acquire your food samples. GMO foods currently approved for sale in the US include corn, soy, papaya, potato, canola, chicory, rice, squash, sugar beets, and tomatoes. However, approval does not necessarily mean that these foods are distributed. The main GMO food crops distributed in the US are corn, soy, and papaya.

As described in the GMO Investigator Kit, different food samples can have widely varying amounts of viable DNA. Please refer to the table in the Tips and Frequently Asked Questions section of the GMO Investigator kit instruction manual for advice on foods that yield good amounts of DNA. Please note that this table only refers to recommendations to obtain good yields of amplifiable DNA; some of the foods may be genetically modified and some may not. In the US, many processed corn-based snack foods or frozen soy-containing meat or vegetarian products do contain GM ingredients. Alternatively, corn or soy standards with specific GM content can be purchased from Sigma-Aldrich or similar chemical supply companies.

Preparing the DNA samples. Follow the extraction procedure outlined in Lesson 1 of the GMO Investigator kit manual for the extraction of DNA from the food samples. Essentially, each sample is ground in water, which is added to InstaGene matrix, heated to 95°C for 5 min, and centrifuged to pellet the matrix. The supernatant of the samples will be used in the PCR.

Planning the reagent requirements. The following should be considered to help plan how to prepare the reagents:

- Determine the number of PCR reactions that need to be prepared. Remember to include replicates
- Determine the number of PCR reactions that will use plant PSII primers
- Determine the number of PCR reactions that will use GMO primers

Bio-Rad's master mix and supermix are provided as 2x (double strength) reagents such that when each is mixed with an equal amount of DNA template it becomes the optimal concentration. A bulk 2x master mix with plant PSII primers (PMM) and a bulk master mix with GMO primers (GMM) will be required for the experiment.

Each PCR reaction volume is 25 µl. Thus each PCR reaction requires 12.5 µl of 2x mastermix composed of SsoAdvanced Universal SYBR® Green Supermix and primers. The amount of bulk 2x mastermix to prepare is calculated by multiplying the number of reactions plus one (to account for pipeting errors) by the volume of 2x master mix required for each reaction.

Calculate how much 2x master mix with plant PSII primers (PMM) and how much 2x master mix with GMO primers (GMM) is required.

The PSII plant primers are supplied at a 25 µM concentration. For this experiment the concentration of PSII plant primers in the 2x PMM should be 0.25 µM. Calculate the volume of PSII plant primers required in the bulk PMM (typically this equates to 1 µl of plant primers for every 100 µl of the 2x PMM plant master mix). Remember the formula $M_1V_1 = M_2V_2$.

$$\frac{(\text{Vol 2x PMM µl}) \times (0.25\ \text{µM})}{25\ \text{µM}} = \text{µl}$$

The GMO primers are supplied at a 100 µM concentration. For this experiment the concentration of GMO primers in the 2x GMM should be 1.0 µM. Calculate the volume of GMO primers required for the bulk 2x GMM as done above (typically this equates to 1.0 µl of GMO primers for every 100 µl of the 2x GMM GMO master mix).

Preparing the PMM. Add the calculated volume of green PSII plant primers to the required volume of SsoAdvanced Universal SYBR® Green Supermix in a labeled screwcap tube. Mix well by pipeting up and down several times and tapping the tube. Spin down to force the solutions to the bottom of the tube and keep on ice.

Preparing the GMM. Add the calculated volume of red GMO primers to the required volume of SsoAdvanced Universal SYBR® Green Supermix in a labeled screwcap tube. Mix well by pipeting up and down several times and tapping the tube. Spin down to force the solutions to the bottom of the tube and keep on ice.

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 instruments's technical support group for additional advice. For advice on the CFX Opus or CFX Duet Real-Time PCR System call 1(800)4-BIO-RAD in the US or contact your local Bio-Rad office.

Plate Setup

See Figures 11 and 12 for a suggestion of 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.

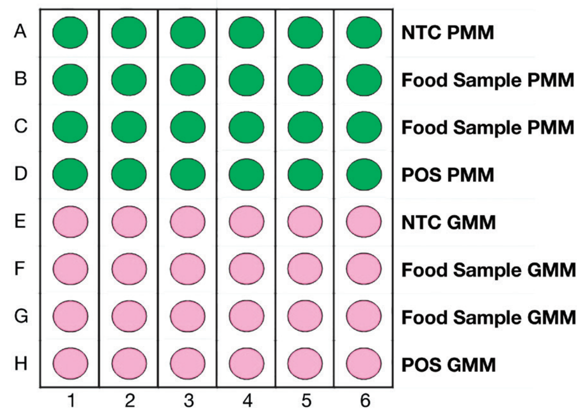


Figure 12. Sample plate setup.

Program 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:	95°C for 4 min	Initial denaturation of DNA
Cycle 2:	94°C for 1 min	Denaturation
	59°C for 1 min	Annealing
	72°C for 2 min	Extension — collect data after this step
	Repeat Cycle 2 for 40 cycles	

Cycle 3 (Optional): 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 sec 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.

Student Protocol

Obtaining Your DNA Templates

1. Obtain your food samples, your negative control, and your positive control.

Preparing PCR Reactions

2. Referring to the plate setup, label the 8-tube strip according to the chosen experimental design to ensure that the PCR strips will be properly identified and oriented. 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 fluorescent reading.

3. Carefully pipet 12.5 µl of PMM to the assigned tubes in the labeled 8-tube PCR tube strips. Make sure to use a fresh tip each time.

4. Again referring to the experimental plan, carefully pipet 12.5 µl of GMM into the assigned tubes in the labeled 8-tube PCR tube strips. Make sure to use a fresh tip each time.

5. Again referring to the experimental plan, carefully pipet 12.5 µl of the appropriate DNA template to the assigned tubes in the labeled 8-tube PCR tube strips. Very gently mix the DNA with the master mix by very slowly pipeting up and down. Do not introduce bubbles into your reactions if possible since these may interfere with data collection by the PCR instrument.

Take care to change pipet tips with each transfer to not cross contaminate samples. Also, make sure not to transfer any of the InstaGene beads from the food samples into the PCR reaction since these beads can interfere with the PCR. One way to help avoid transferring the InstaGene beads is to pipet from the top of the sample since the beads have been pelleted to the bottom of the tube. If the beads are disrupted, centrifuge the DNA samples to repellet the beads.

6. Once reactions are mixed, cap the tubes and spin down the PCR tubes to force the reactions to the bottoms of the tubes and to dissipate bubbles (10 sec at full speed).

7. Place your PCR strip tubes in the instrument, ensuring that the location of the wells exactly matches that specified in the plate setup.

8. Make sure that all lids of the strip tubes are securely fastened to avoid any evaporative loss. Check to make sure that the optical faces on the caps are clean.

Running PCR Reactions

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

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

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

Gel Electrophoresis Analysis

Remember that real-time PCR gives specific information about the quantity of starting template, whereas agarose gel electrophoresis gives specific information about the size of the amplicons produced. The plant primers in the GMO kit will produce an amplicon of approximately 455 bp and the GMO primers will produce 1–2 amplicons of approximately 203–225 bp. However, in the absence of starting template those same primers will produce a much smaller primer-dimer amplicon. Additionally, there is a significant variety of genomic DNA present in your test food DNA samples. You may see non-specific PCR bands that are visible on an agarose gel and in melt-curve analysis.

Real-time PCR plots for the true PCR product and for the artifact primer-dimers and non-specific amplicons look very similar, yet the band pattern for these two different amplicons looks dramatically different on an agarose gel. Therefore, running an agarose gel of the PCR products is highly recommended — so that the user may gain experience in interpreting real-time results that may contain both true product (plant or GM amplicons) and artifacts (primer-dimers and non-specific amplicons).

12. Prepare 3% agarose gels as described in the GMO Investigator Kit protocol, with enough lanes for the analysis of at least one strip of reactions plus a lane for the PCR molecular weight ruler (this would require 9 lanes).

13. Add 6 µl of Orange G loading dye (provided in the GMO Investigator Kit) to each 25 µl PCR reaction that will be loaded on the gel.

14. Add 50 µl of Orange G loading dye to the vial of PCR molecular weight ruler. Mix well and pulse-spin.

15. Load 20 µl of each of the PCR samples, as well as 20 µl of the PCR molecular weight ruler on the agarose gel.

16. Run the agarose gel at 100 V for 30 min. Analyze the results as described in the GMO Investigator Kit protocol.

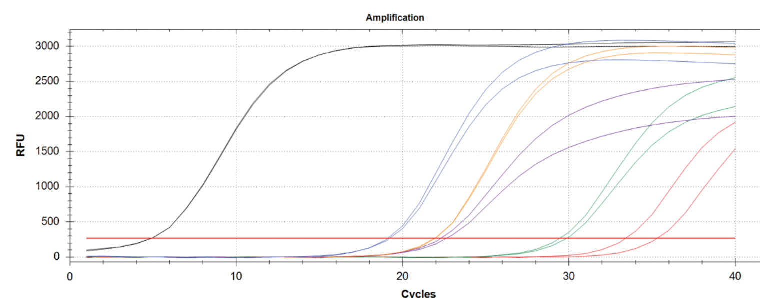
Laboratory 2 Results and Analysis

After completing the Laboratory 2 protocol, a set of real-time PCR data and an agarose gel will need to be analyzed. Below is some information and some suggestions to help with the data analysis.

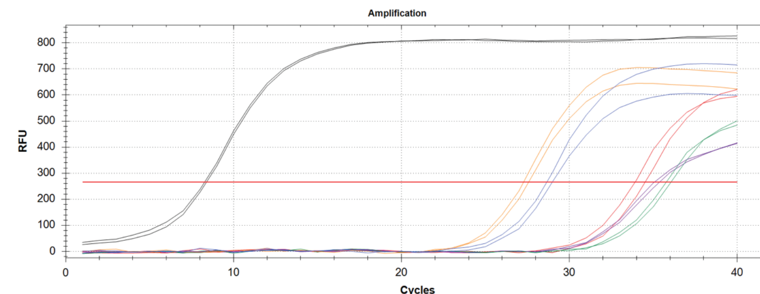
Set the Threshold Level. The real-time instrument software will automatically set a threshold level. Verify that this is in the correct position by ensuring the threshold line is above the background noise of the no template control and in the exponential amplification phase of the samples. The figure below shows the proper placement of a threshold line.

SsoAdvanced – Duplicate samples

Higher RFU = Plant Primers



Lower RFU = GMO Primers



PMM	GMO
Pos	Pos
NTC	NTC
Banana	Banana
Grain	Grain
Corn Chip	Corn Chip
Papaya	Papaya

Figure 13. Amplification of food samples with PSII plant and GMO primers. Duplicate samples: GMO-positive control DNA (Black/ Gray), no template control (NTC) (red/ pink), banana (olive/ yellow), grain (green/ light green), corn chip (orange/ light orange) and papaya (blue/ light blue). Amplified with plant primers (first listed color) or GMO primers (second listed color). The traces indicate the amplification of the positive control DNA with PSII plant and GMO primers had Cq values of ~5 and ~9, respectively. Thus, it took 5 PCR cycles for the level of fluorescence (and therefore the quantity of PCR product) to reach the quantitation level for plant primers and 9 cycles using GMO primers. The NTC controls reach the threshold line around 35 cycles for both primers. The traces with plant primers for the banana, grain, corn chip, and papaya have C_q values of ~22, 29, 22, and 19, respectively. All are well above the NTC C_q value. The traces with GMO primers for the banana, grain, corn chip, and papaya have C_q values of ~35, 35, 27, and 29, respectively. This indicates successful amplification of GMO amplicon from the corn chip and papaya food samples while banana and grain samples matched the C_q of the NTC.

Please note that it may be easier to set the threshold line using the log view that changes the y-axis of fluorescence units to a logarithmic scale. Consult your real-time instrument's manual for additional information on setting the threshold level.

Melt-Curve Analysis. Verify that the C_q values are from genuine PCR products and not from primer-dimer signals. Analyze your melt curves against the positive control. If the sample has a peak that does not match the positive control, and matches a peak in the NTC, then the target amplicon may not have been amplified; any signal may have derived from primer-dimers. Use your agarose gel data to confirm that the target amplicons have been amplified.

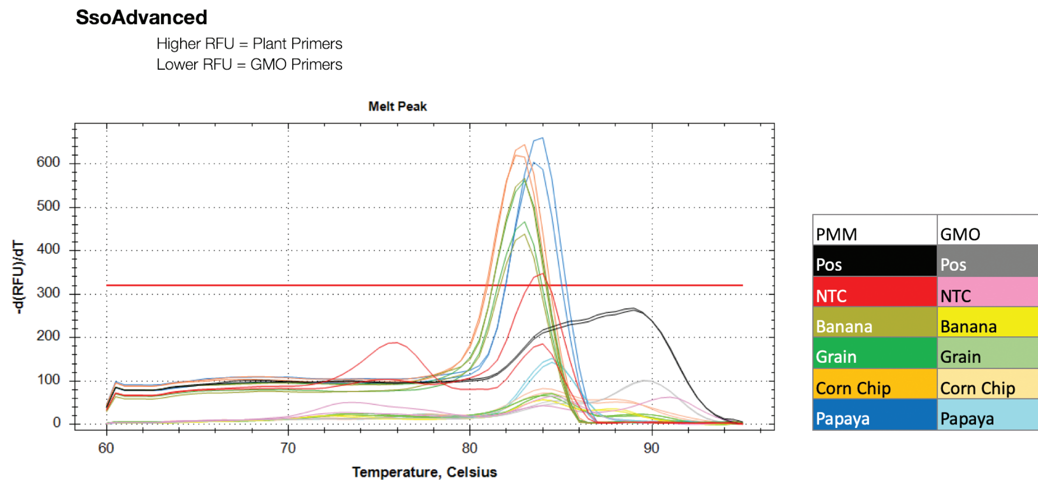


Figure 14. Melt-curve analysis after amplification of food samples with GMO primers. Duplicate samples: GMO-positive control DNA (Black/ Gray), no template control (NTC) (red/ pink), banana (olive/ yellow), grain (green/ light green), corn chip (orange/ light orange) and papaya (blue/ light blue). Amplified with plant primers (first listed color) or GMO primers (second listed color). The major peak for the GMO-positive control is at 84°C, this correlates with peaks for the papaya and corn chip samples, indicating this peak represents the GMO PCR amplicon. This information should be confirmed on an agarose gel.

Obtain C_q values for samples. The cycle of the PCR reaction at which the relative fluorescence of the sample crosses the threshold line is referred to as the C_q value. These will be calculated by the real-time software and can also be visualized from the real-time graph.

Generate a table for the data with the following headings: Sample name, PSII Plant primers, and GMO primers. Fill in the C_q values for each sample. If replicate reactions have been performed as a class or for individual teams, calculate the average C_q value and the standard deviation for each sample. It may be possible to program the real-time software to automatically process replicates — please consult the instrument manual.

	Cq	
	Plant Primers	**GMO Primers**
GMO Positive Control	5	9
NTC	35	35
Grain	22	35
Banana	29	35
Corn Chip	22	27
Papaya	19	29

Figure 15. C_q values from the experiment noted above. The fluorescence of the corn chip snack PCR reaction with plant primers reached the threshold line after the papaya (at cycle 22 vs. cycle 19), indicating more total plant DNA was extracted from the papaya snack than the corn chip snack. However, the fluorescence of the corn chip PCR reaction with GMO primers reached the threshold line before the papaya (at cycle 27 vs. cycle 29), indicating more GMO DNA was extracted from the corn chip snack than the papaya. The grain and banana PCR reaction with the plant primers reached threshold at cycles 22 and 29, comparable to corn chip and papaya.However, the PCR reactions with the GMO primers reached threshold at cycles 35 for both, comparable to NTC. Which of these samples has the most GMO DNA present?

Evaluate the results of your C_q values. Do the positive and negative controls show the expected C_q values? Which of the food-extracted DNAs fall within the range of the NTC and the positive control DNA C_q values? Compare these C_q results with the gel electrophoresis results. Do they agree?

Analyzing the agarose gel. The GMO Investigator Kit is traditionally analyzed based on the agarose gel results. Since the plant primers generate a PCR product of 455 bp, while the GMO primers generate a product(s) of 203–255 bp, the presence or absence of GMO content can be deduced from the agarose gel results.

Which of the samples had successfully amplified plant DNA? From which foods was there successfully amplified GMO positive DNA?

Generate a table containing the sizes of your amplicons and their relative intensities.

Does this gel provide information on the relative amounts of starting DNA template? If so, what information can be obtained?

How does this information correlate with the melt-curve analysis?

Note how the quantitative real-time PCR and the agarose gels provide very different, but equally valuable, pieces of information. Consult the GMO Investigator manual for further assistance analyzing the agarose gel results.

Quantitate food DNA relative to known control DNA. It is possible to use real-time data to obtain quantitative information on the DNA concentration of the unknown samples without a standard curve. The concentrations are derived as a ratio relative to the DNA content in the other samples. The ratio of templates from the C_q values can be calculated because the C_q values are related to the initial template concentration. Remember that each PCR cycle doubles the amount of PCR product, so after three cycles if there was y starting number of templates, there would be y x 2 x 2 x 2 templates, or y x 2^3 templates.

The C_q value represents the number of cycles it takes each PCR reaction to reach a specific level of fluorescence. For example, the C_q value is also the cycle at which each PCR reaction reached a threshold quantity of PCR products. For example, if sample A had 8 times more starting DNA (16,000 copies of template) than sample B (2,000 copies of template) and the threshold level was set to the equivalent of 64,000 copies of template, then sample A needs only two cycles to reach 64,000 copies and has a C_q value of 2 while sample B needs five cycles (2,000 x 2^5) and has a C_q value of 5.

The relationship between copy number and C_q value is inversely related: the higher the C_q, the lower the copy number of starting template and since the cycling reaction causes template to double, the relationship is also logarithmic:

Copy number is proportional to 2^{-C_q}

The role of the standard curve is to determine what “is proportional to” actually means. In the absence of a standard curve, though, it is possible to compare copy number ratios by comparing their C_q values. Remember that the operation for division of logarithmic numbers is to subtract exponents, so for example:

$$2^{-C_q\,(\text{control DNA})} / 2^{-C_q\,(\text{test DNA})} = 2^{-[C_q\,(\text{control DNA}) - C_q\,(\text{test DNA})]} = \textbf{Ratio of control DNA templates to test templates}$$

In the experiment above, the ratio of starting DNA template from the GMO-positive control DNA to the corn chip snack is

$$2^{-(9-27)} = 2^{18} = 262{,}144$$

Thus the GMO-positive control DNA sample had 262,144 times more starting DNA than the corn chip snack DNA sample.

Using the above information and equations, derive the ratio of GMO-positive control DNA to PSII plant templates for each of the food samples tested.

How many more GMO-positive DNA templates were there compared to food DNA templates?

Look at the agarose gel. Would it be possible to have deduced the result above from the agarose gel data?

Comment on the value of conventional PCR analyzed by agarose electrophoresis versus the value of real-time PCR analysis.

Quantitate GMO content relative to a reference gene. Real-time PCR is often used to test the level of a target sequence that is variable compared to the total amount of DNA/RNA in a sample. For example, real-time PCR may be used to test whether the expression level of mRNA of a specific gene is increased in cancer cells versus normal cells or, as in this experiment, what the relative GMO content of one food is compared to another. The level of the specific variable target sequence is measured relative to a target sequence that does not vary between samples — a reference gene. The amount of variable target is first normalized against the reference gene to level out differences in starting quantities of template and then the relative levels of the variable target between samples are derived. In this case the variable GMO content of foods is being investigated. The total DNA content between the two food samples will be normalized using the PSII gene as a reference and then the relative levels of GMO content between the food samples will be calculated.

From the example above, the GMO content of each sample is first normalized against the PSII plant gene for both snacks. This is done in the same manner as the ratio of DNA quantity between samples was derived above, except in this case, the ratio of two different genes (GMO and PSII) from the same sample rather than the ratio of the same gene in two different samples is derived.

Sample	Cq (Plant)	Cq (GMO)	Cq (Plant) - Cq (GMO)	Ratio of GMO to Plant Copies
Corn Chip	22	27	-5	3.13%
Papaya	19	29	-10	0.10%

The data above shows how much GMO template was present in the starting template relative to the PSII template. In the corn chip there was 32 times more PSII template than GMO template (100/3.1%), while in the papaya there was 500 times more plant DNA than GMO DNA.

Next derive the ratio of GMO content between the samples.

$$\text{Ratio of GMO content of corn chip over papaya} = \frac{\text{Ratio of GMO/Plant for corn chip}}{\text{Ratio of GMO/ Plant for papaya}} = \frac{3.12}{0.10} = 32$$

Thus the corn chip has 32 times the GMO content of the papaya.

Using your own data, normalize the GMO content against the PSII gene for each food sample.

$$\frac{2^{-C_q\,(\text{GMO})}}{2^{-C_q\,(\text{plant})}} = 2^{-[C_q\,(\text{GMO})\,-\,C_q\,(\text{plant})]} = \text{Ratio of GMO to plant genes}$$

Do this for each sample.

Derive the ratio of the ratios between two food samples.

$$\text{Ratio of GMO content of food 1 over food 2} = \frac{\text{Ratio of GMO/PSII for food 1}}{\text{Ratio of GMO/PSII for food 2}}$$

Please note that this ratio makes the assumption that the number of copies of PSII genes per cell (for example, the number of chloroplasts per cell) is consistent between both foods. This may not be the case. A better reference gene would be a single copy reference gene from nuclear DNA rather than the chloroplast DNA.

Conclusions and Ideas for Further Exploration

In addition, this analysis assumes the occurrence of GMO target sequences is identical between samples. Many GM crops are compounded; for example, are generated by crossing two GM crops with different modifications. Rather than testing for a specific inserted coding sequence, such as the CRY1 gene in Bt corn, the GMO Investigator kit and real-time application assays for common promoter and terminator sequences which may be present multiple times within the same genome. As such, this activity works well to teach the principles of quantitative real-time PCR analysis of genetically modified foods. However, for more precise quantitation, research laboratories often quantify the number of copies of a specifically inserted gene such as CRY1. For further information, please refer to Bio-Rad's Real-Time PCR Applications Guide, Rev B for more detailed information on GMO detection including experimental design and data analysis.

What conclusions can you draw from your results? Some key things to evaluate in your dataset from the DNA extract data are:

- **Consistency of replicates.** Look for problems in duplicate sets of each PCR reaction and try to troubleshoot what may have gone wrong. Calculate the average C_q values for each replicate.

- **C_q values.** Do the positive DNA controls and the negative control show the expected C_q values? Which of the food-extracted DNAs fall within the range of the NTC and the control DNA C_q values? Compare these C_q results with the gel electrophoresis results. Do they agree?

- **Melt-curve and electrophoresis results.** Does the DNA gel banding pattern correspond to what you expect from the melt-curve analysis? Which samples had primer-dimers? Were the GMO and plant amplicons of the expected sizes?

Quantitate the DNA in the samples using a standard curve. The standard curve generated in Laboratory 1 can be used to estimate the relative quantity of DNA target in the unknown samples by comparing the C_q values from the unknown samples on the x-axis with the corresponding DNA quantity on the y-axis of the standard curve. This can be done separately for the plant and GMO reactions and will allow the relative quantitation of GMO to plant DNA to be calculated for each sample.

Please note that normally the standard curve would be run in the same experiment as the unknown samples to control for experimental variability. However, in this exercise the activities were separated in order to simplify the experiment. Since the threshold line is arbitrarily assigned, it may be necessary to adjust the level of the threshold line of either or both of the experiments such that the C_q values of the same concentration of GMO positive control DNA are comparable between the experiments.

Tip: If the real-time PCR software, rather than a graphing program, was used to generate a standard curve, the line equation for the C_q values may generate base-10 logarithms that need to be converted back to actual C_q values.

Compare the results from the absolute quantitation with the standard curve with the relative quantitation using the formulas above. Do the data correlate?

Laboratory 1 in this application note demonstrates the quantitative nature of real-time PCR and Laboratory 2 puts the process into practice by evaluating the DNA content of actual foods. After analyzing food samples, real-world quantitative PCR calculations can be used to interpret the results, which will give interesting information about the relative amount of DNA recovered from different samples. It is even possible to determine the percentage of GMO content in a particular food. Comparison of real-time data with traditional agarose gel electrophoresis demonstrates the very different types of information that these techniques provide.

For individuals wishing to continue these experiments further, there is a wide range of activities that could follow on from these laboratories. For example, a discovery approach can be included with the following types of scenarios:

Regulatory concerns over GMOs. Real-time PCR is playing a central role in a longstanding dispute between the European Union (EU) and the USA about the labeling of GMO foods, a dispute that lends itself well to a class study of many different topics relevant to the biotechnology industry. The scope of this problem is large and growing, since the prevalence of GMO crops grown each year in the USA has increased to the extent that the major crops such as corn and soybeans are substantially GMO. Segregation of non-GMO crops from GMO crops is not an easy solution, since neither the grain harvesting and distribution infrastructure or the food production system easily lend themselves to the segregation of one type of seed from another. Even the biological system of plants (for example, pollenation) stymies attempts to segregate GMO from non-GMO crops. The dispute between the EU and the USA on GMO foods provides the biotechnology class with a variety of interesting and relevant topics such these:

- The impact of the new GM crops on international trade. Intolerance to GMO led to an outright ban of all GMO foods from the EU in 1999.

- How GMO foods are regulated. The zero tolerance to GMO foods by the EU was lifted in 2003 and replaced with new regulations that carefully define what is GMO and what is not, as well as how they are to be detected, traced, and labeled. Under current EU legislation, all GMO foods require authorization by EU regulatory systems following safety testing, and authorized GMO foods must have specific DNA sequences that act as identifiers for traceability of the transgene. Authorized GMO foods are required to be labeled if they exceed a 0.9% ratio of GM to plant reference gene copy number.

- How real-time PCR is used to detect and trace GMO foods by the EU. Real-time testing provides a reliable way of certifying that a food does not exceed the EU threshold limit for being classified as a non-GMO product. Under current EU legislation, all authorized GMO foods must have a specific GM DNA sequence that acts as an identifier for detection and traceability of the transgene. Students can research what reference DNA materials have been developed by the Institute for Reference Materials and Measurements (IRMM) of the European Commisions' Joint Research Centre (JRC) for specific GMO crops. They can go on to purchase these reference materials to more closely model a regulatory laboratory and assay specific GMO foods for labeling requirements, following the exact certified procedures required.

- Effects of international law on regulatory decisions. The most recent ruling that requires labeling of GMO crops in the EU has led to litigation by the USA through the WTO. The USA leads the world in production of GMO crops, and only six countries worldwide account for 99% of the GMO crops planted each year. Arguments are being made that the EU regulation of GMO foods is discriminatory, amounting to a trade war. At the heart of these arguments is the premise that GMO foods are safe and do not cause health problems, an idea that is being challenged by some scientists. This is another topic that students may want to explore.

Some good starting points for developing class projects involving the EU labeling regulations include:

- Real-time PCR technology and primers used for quantifying GM transgenes in specific species:

  Holst-Jensen A et al. (2003). PCR technology for screening and quantification of genetically modified organisms (GMOs). Anal Bioanal Chem 375, 985–993.

  Levin, R.E. (2004). The Application of Real-Time PCR to Food and Agricultural Systems. A Review. Food Biotech 18, 97–133

  Markoulatos, P., et al. (2004). Qualitative and Quantitative Detection of Protein and Genetic Traits in Genetically Modified Food, Food Rev Int. 20, 275–296

- How real-time PCR is used to detect and trace GMO foods by the EU:

  Sustainable Introduction of Genetically Modified Crops into Euraopean Agriculture. **https://cordis.europa.eu/project/id/501986/reporting**

- Reference materials for GM crops regulated by the EU:

  Institute for Reference Materials and Measurements (IRMM) of the European Commisions' Joint Research Centre (JRC): **https://crm.jrc.ec.europa.eu/**

DNA quantitation. How much DNA comes from different food types? Since quantitative PCR clearly identifies the relative amount of DNA recovered, a wide range of foods could be sampled and analyzed using the plant primers alone. Do frozen peas have different DNA content from fresh peas? Do organic carrots have more or less DNA than regular carrots? Does salad oil truly have virtually no DNA? Such investigations will help students to learn about the effects of food processing on the levels and integrity of its DNA content.

DNA extraction efficiency. Do different methods of DNA extraction improve quantitative PCR results? The Instagene matrix used in the GMO Investigator kit is a rapid method for extracting DNA suitable for PCR, but it is not optimized for collecting large quantities of pure DNA. Traditional DNA extraction methods (lysis, boiling, precipitation) can often yield far more DNA. How does this affect quantitative PCR? Is it easier to quantitate GMO content if more starting DNA is available?

Design your own assay. Can you quantitate your own genes? There are a number of freely available on-line primer design tools (for example, Primer3 at **http://primer3.ut.ee**) which can be used to design PCR primers and these can ordered from a number of on-line primer supply companies. You could design primers for any gene of interest and analyze them by quantitative PCR. The possibilities are limitless — you could quantitate any gene you could imagine using this technique.

This application note is just a starting point for quantitative PCR. There is much room for experimentation and adaptation, so have fun with your adventures in real-time PCR!

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 $\%E = (E-1) \times 100\%$, 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_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 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 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”.

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.

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.

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.

SsoAdvanced Universal SYBR® Green Supermix It is an exclusive high-performance real-time PCR reagent based on Bio-Rad's patented* Sso7d fusion protein polymerase technology and advanced buffer formulation.

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.

* U.S. patent 7,560,260B2.

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 acitivty, 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.

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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