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BE493
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qPCR Final Report

Introduction – Theory and Practice

Quantitative Polymerase Chain Reaction (qPCR) is a cornerstone technique in molecular biology, enabling the precise quantification of nucleic acids to be monitored in real time. (link quantitative pcr basics) In this section, we delve into the practical and theoretical aspects of using a qPCR instrument and the process step by step from execution to analysis.

An essential prerequisite to a qPCR is the preparation of a high-quality DNA sample. Nucleic acids are derived from different biological samples including, but not limited to cells, tissues, and blood. The DNA is then purified to remove any potential contaminants that could disrupt the readings. The next step is primer design, where the specific primers used to anneal to the target sequence are chosen. Lastly, a probe or dye is selected depending on the goals of the experiment. (link pcr basics)

The difference between using a dye and probe in the machine is that dyes are intercalating which means the molecules are inserted between the planar bases of the DNA while the fluorescent probe depends on fluorescence quencher molecule which is covalently bound to the probe (link qpcr anaysis). Fluorescence quencher molecule decreases the fluorescence intensity. For this reason, probes are sequence specific so each probe must be designed for each experiment, improving assay specificity but increasing cost and time. The qPCR machines may include various filters to receive multiple wavelength signals from fluorescence emitted by the probes allowing for the performance of multiple tests in a single well. Unlike probes, since dyes are not sequence specific, it will fluoresce whenever a DNA amplification occurs (the relationship is proportional) so muxing is not possible. Multiple wells must be used for the experiment, each with varying targets and its own primers.

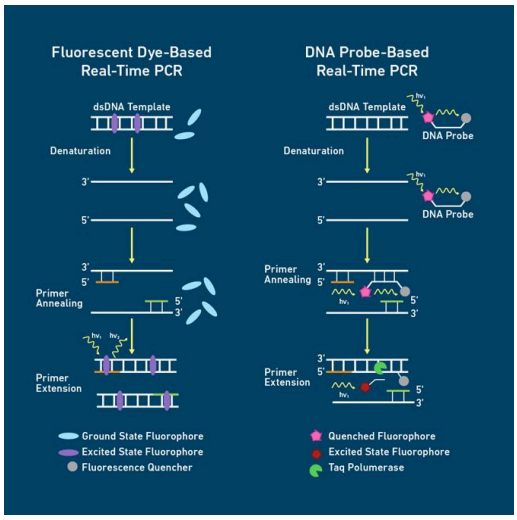


Figure 1: Illustration of dye versus probe based qPCR

After the DNA is prepped, the primers selected, and the dyes chosen, the mixture that will undergo the heating cycles in the qPCR is created. This solution includes the sample DNA, the primers, DNA polymerase, the probe or dye, and nucleotides (dNTPs). (link pcr basics) This mixture is then pipetted into either a qPCR plate and covered with a plastic film to prevent evaporation or into PCR tubes and loaded into the qPCR machine, ready to be heated.

In this experiment, SYBR Green was added to the qPCR mix and is used to quantify the double stranded DNA. When SYBR Green binds to the DNA, it fluoresces, and the corresponding fluorescence emission is quantified using the following optical subsystem (Figure 2) where the light source provides the excitation light, filters selectively transmit certain wavelengths, and the detectors capture the emitted fluorescence. The intensity of the fluorescence is used to determine the quantity of DNA. (link introduction to qpcr system) Graphically, this intensity is noted as the relative fluorescence unit (RFU) and is graphed against the cycle number.

A standard qPCR reaction takes 1 hour 45 min starting with the initial denaturing where the hydrogen bonds between the DNA base pairs are broken creating single stranded DNA from

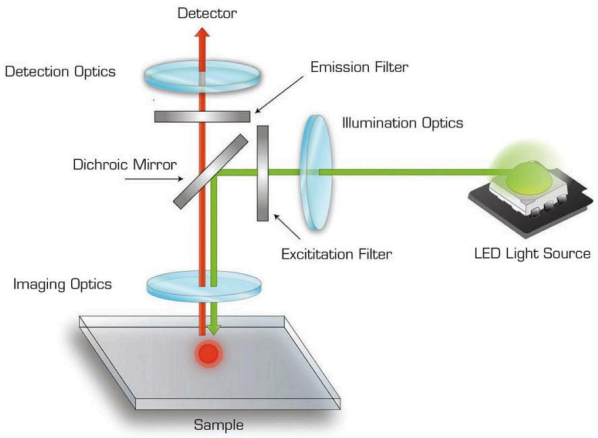


Figure 2: Diagram from Bio-Rad's Introduction to qPCR System of a typical optical detection system in a qPCR system consisting of the illumination source, illumination optics, collection optics, detection optics, excitation filters, and emission filters. link

the sample double stranded. During the initial denaturing the temperature is increased to 95 deg C incubated for 2-15 min depending on the polymerase hot start mechanism being activated. (link pcr basics) The resulting single stranded template DNA is now ready to base pair with the primer.

The next step is annealing where the temperature is typically lowered to 60 °C for 30 seconds. The drop in temperature allows the primers to bind with the complementary

sequences in the template DNA. The temperature at which this is done is very important as it ensures that the hybridization only occurs when the primer matches the target

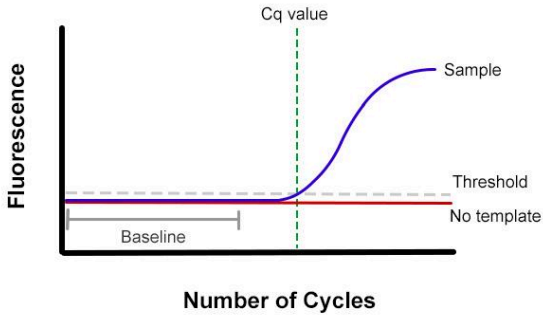
cr)

Once the primer is bound the next step is elongation where the temperature is increased to 72 °C and held for 30 sec per 1 kb of DNA. During this time the DNA polymerase synthesizes a new DNA strand that is complementary to the template DNA by extending the primer and adding dNTPs to the 3' end of the strand. (link pcr basics) The SYBR Green binds to the newly created double stranded DNA (dsDNA) and fluoresces. This fluorescence is detected by the fluorimeter within the qPCR machine and recorded within the software. The denaturing, annealing, and elongation are typically 30 times as this will result in the sample DNA being exponentially amplified resulting in 2^{30} times the sample and 10^9 copies. (link what is real time qpcr)

To achieve these exact temperatures a thermal cycler, specifically the VeriFlex blocks in the QuantStudio 5 qPCR machine, is used, which automates the temperature cycle and incubation times. (link pcr basics) The qPCR plate or tubes are placed directly in the block and cyclically heated.

As previously mentioned, the quantity of DNA is determined by the intensity of the SYBR Green emissions. When plotted against cycle number, this results in a graph with two distinct phases, an exponential phase then a non-exponential phase that plateaus. During the exponential phase, the amount of DNA theoretically doubles each cycle, but as the reaction proceeds and components are consumed the reaction slows and enters the plateau phase. (link what is real time qpcr) This relationship is shown below in Figure 3 below.

When interpreting the amplification curve there are three critical values that must be determined which are shown in Figure 4. The first is the baseline, which is, as the name suggests, background fluorescence signal unrelated to the sample caused by external factors. The baseline is determined in the initial cycles (cycles 3-15). (link what is real time qpcr) Next, is the threshold value which is a fluorescence value that represents a significant departure from the baseline. The quantstudio 5 automatically sets a



threshold for simplicity. Lastly is the Ct value (Cq in Figure 4) is the number of cycles at which the fluorescence signal reaches the threshold. The Ct is particularly useful because of its inverse relationship with the starting amount of target DNA. (link what is real time qpcr)

With the possibility of various sample qualities and unknown PCR efficiency, normalization of the gene expression is crucial. This is done by calculating the difference in average Ct, ΔCt (link steven). The ΔCt equation is given as:

$$\Delta Ct \;=\; average\ Ct\ from\ one\ dilution \;-\; average\ Ct\ from\ other\ dilution$$

Calculating ΔCt ensures that the observed changes in the experiment are specific to the gene analyzed and not due to external factors.

To quantify the change in gene expression of the treated sample to the control, ΔΔCt is calculated (link steven). ΔΔCt is given by:

$$\Delta\Delta Ct \;=\; \Delta Ct(treated\ sample) \;-\; \Delta Ct(untreated\ sample)$$

Then you can take the ΔΔCt and put negative of it to power of 2 such that (link steven):

$$Relative\ fold\ gene\ expression\ level \;=\; 2^{-\Delta\Delta Ct}$$

However, in this experiment, the samples are not treated. Therefore, the equation $2^{\Delta Ct}$ is used to determine fold change because the samples (excluding water) are the same solution at different concentrations.

The amplification can be further analyzed using standard curves and melting curves. A standard curve is constructed with known DNA concentrations and Ct values from unknown samples can then be interpolated from the curve. (Link explaining multiple) Because SYBR Green non-specifically binds to double stranded DNA to double check that a single target was amplified a melting curve is done at the end of the PCR process. During melting curve analysis the temperature is increased from 60°C to about 95°C to denature the DNA. The derivative of the resulting decrease in fluorescence is then plotted and if there is one peak it is likely that only a single product was amplified. (Link explaining multiple)

Use Cases

Quantitative PCR (qPCR) is used to amplify and quantify a predetermined target in a sample, which is useful in many different fields such as diagnosis of bacterial infections, detection of genome-edited organisms, and detection of mutations present in cancer. While qPCR performs the same function in all of the above examples, the data gained from qPCR is useful in a variety of applications.

The first paper to be reviewed involves the use of qPCR in diagnosing skeletal bacterial infections of *Mycobacterium tuberculosis* (MTB). The goal of this paper was to determine whether or not qPCR was a viable option for the diagnosis of skeletal MTB, and to compare it to current diagnosis methods like acid-fast bacillus staining (AFS). (link he)In this paper, DNA was extracted from skeletal tissue to be used with a commercial qPCR kit to prepare the samples for qPCR, and primers were designed for the MTB targets. The qPCR cycling conditions were created as follows (per manufacturer's instructions) and run in the qPCR:

1. 95 °C 5 min → 2. 95 °C 15 s → 3. 72 °C 30 s → 4. Repeat steps 2-3 45x → 6. 60 °C 30 s

Importantly, the cycle threshold values were a fixed fluorescence threshold to determine positive or negative results.

Once the qPCR assay was completed, statistical analysis was done to compare qPCR results with AFS results in the diagnosis of skeletal MTB. It was found in this paper that qPCR

was better than AFS at accurately diagnosing skeletal MTB in both sensitivity and specificity. (link he)

The second paper to be reviewed describes the use of qPCR in detecting the first commercialized genome-edited crop to suggest its applicability in any future genome-edited organism. In this paper, the qPCR was used to detect a single-nucleotide genome edit in canola. (link Chhalliyil) Primers were created to detect this genome edit, and the qPCR was done using TaqMan probes for detection with cycle conditions as follows in the qPCR:

1. 95 °C 10 min → 2. 95 °C 30 sec → 3. 60 °C 1 min → 4. Repeat steps 2 - 3 45x

The data collected from qPCR was used to distinguish the mutation of the gene-edited canola from different variations of unedited canola. The primers used in the qPCR detected the mutation (or ignored the lack of mutation) for all canola variations to determine this difference. This experiment was repeated for varying concentrations of gene-edited canola to understand the extent to which the qPCR could amplify and detect the difference between edited and unedited canola. These results were analyzed through the amplification curves and finding the mean Ct value for the varying canola types to show the differences, and conclusions were made about future use of qPCR for detecting mutations in genome-edited crops. (link Chhalliyil)

The third paper to be reviewed covers the use of qPCR to detect breast cancer as a diagnostic tool. The purpose of qPCR in this paper is similar to the first paper, but for breast cancer cells in tissue as opposed to infectious skeletal TB cells. Additionally, since this paper is not infectious, it shows promise for quantifying differences in human mutations using allele-specific amplification of targets (targets being the two mutation variations). This study used primers specific to the various mutations, and used a Qiagen kit to carry out the DNA extraction and qPCR preparation with SYBR Green for detection. The cycle conditions used in the qPCR are as follows:

1. 95 °C 10 min → 2. 95 °C 15 sec → 3. 60 °C 1 min → 4. Repeat steps 2 -3 40x

In this experiment, the data from the qPCR included a melting curve analysis to determine what was amplified, and a comparative cycle threshold analysis was conducted. By analyzing significant differences between amplification of samples to the control samples, positive and negative diagnoses were able to be made for a common mutation that causes breast cancer. This presents the possibility for future lower cost diagnoses of common cancers through qPCR. (link alvarez-garcia)

Materials and Methods

For the experiment, the qPCR protocol document from BlackBoard was referenced. To set up the qPCR run, two samples were created. Using a mixture of transcription factors for E.coli DNA and SYBR Green Master Mix, named PCR1, different concentrations were created through dilution with deionized water. Forward and reverse primers that match the transcription factors are also needed in the mix, but due to improper training, the primers were not added into the PCR1 mixture. For the first dilution, 25µL of PCR1 and 50 µL of deionized water was added to a microcentrifuge tube using a 20-200 microliter pipette. For the second dilution, 50 µL of PCR1 and 25 µL of deionized water was added to a different microcentrifuge tube. After the solutions were added, both of the microcentrifuge tubes were mixed before pipetting into a 96-well plate, compatible with the QuantStudio5 qPCR machine. It is important that the 96-well plate fits into the machine, to avoid the plate potentially getting stuck.

The 96 well-plate had triplicates of each sample, PCR1 without any dilution, as well as deionized water. So, in total, there were four different types of samples in the well-plate. In Row A, columns 1-3, 25µL of deionized water was pipetted into the plate using the 20-200 microliter pipette, totaling 75µL across Row A. The same procedure followed for the two diluted samples

and PCR1. Row B contained the first dilution (25µL of PCR1 and 50 µL of deionized water), Row C contained the second dilution (50 µL of PCR1 and 25 µL of deionized water), and Row D contained only PCR1.

After the plate has all of the samples, a heat reactive adhesive was placed over the well-plate. Once the well-plate was ready, it was placed into the drawer of the QuantStudio5 machine and closed by clicking a button on the top-right corner of the machine screen. Through the laptop that is connected to the qPCR, the template, "be493_qpcr_template_2024.edt," was opened and renamed to distinguish it from the other qPCR experiments. In the template, the samples were defined based on well-position – sample 1, sample 2, sample 3, and water control – with the same target (Target 1). Once everything was assigned, the qPCR started where it took approximately one hour and 15 minutes to complete, using the following heating process:

1. 95 °C 2 min → 2. 95 °C 10 seconds → 3. 55 °C 30 seconds → 4. 72 °C 30 seconds → 5. Repeat steps 2-4 30 times → 6. 72 °C 60 seconds

When the qPCR was finished, the experiment file was exported using Excel. In the Excel spreadsheet, there are tabs at the bottom of the spreadsheet labeled Sample Setup, Amplification Data, Results, Melt Curve Raw Data, and Melt Curve Result. Using the amplification data, an amplification plot was created for the three samples, with the x-axis in cycles and the y-axis in the change in R_n, where R_n is the fluorescence intensity.

To further analyze and quantify the gene expression, the Ct difference was calculated for each concentration of the DNA sample using the formula:

$$Ct\ difference\ =\ average\ Ct\ from\ one\ dilution\ -\ average\ Ct\ from\ other\ dilution.$$

Ct is the number of cycles where the qPCR curve crosses the automatic threshold set by the QuantStudio5 machine (Figure 6). The threshold in the experiment was 0.200, and it is where there will be a significant detection of fluorescence that is different from background noise. The Ct averages of the dilutions have already been calculated in the Results section. For the expected Ct averages, a high concentration of DNA should have a lower Ct since amplification happens earlier, and it should have reached the threshold at an early cycle (link Understanding qPCR results).

Setting the original concentration of the DNA as the reference concentration, an example of the Ct difference calculation would be:

$$Ct\ difference\ low\ concentration\ =\ Ct\ average\ original\ -\ Ct\ average\ low.$$

The Ct difference value would then be used to calculate the fold change = $2^{\text{Ct difference}}$. Depending on the value of the fold change, it can determine how much more or less DNA was amplified compared to the original concentration reference. For the expected fold change, as one increases the concentration of DNA, there should be an increase in the fold change.

Results

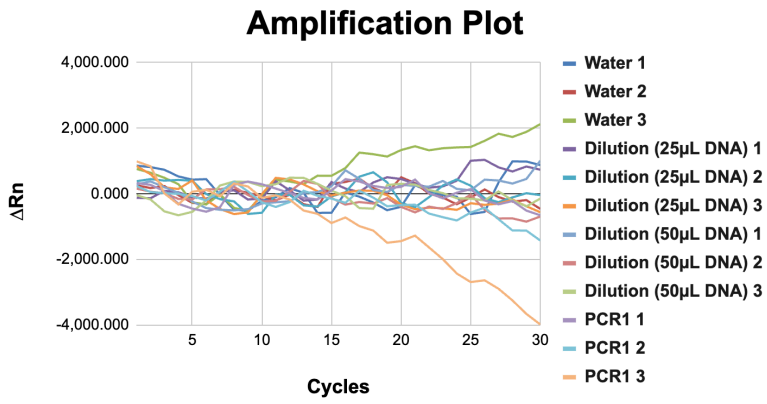


Figure 7: Amplification plot of PCR solutions at varying dilutions and water without primers

For the experimental data, there was an expectation to see exponential growth for the

DNA samples. However, as shown in Figure 7, there was only a slight growth for water. SYBR green was not added to the water control, so there should have been no amplification detected. In addition, PCR1 3 had negative fluorescence emission. As a result, these results are determined to be inconclusive.

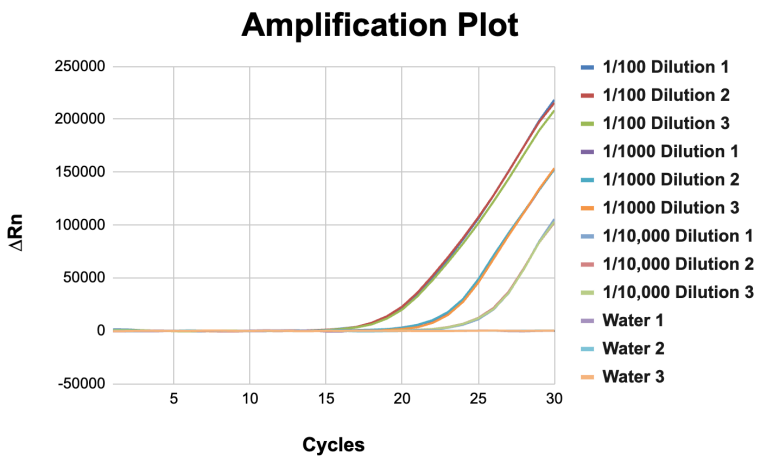


Figure 8: Amplification plot of PCR solutions at varying dilutions and water with primers

In Figure 8, the graph illustrates a logarithmic curve of sample data that represents the exponential relationship between DNA amplification and DNA concentration. At higher DNA concentrations, it displays a higher amount of amplification in fewer cycles. Water displays no trend and stays constant throughout the experiment at zero ΔRn, differing from Figure 7.

Table 1: PCR solution concentration and corresponding cycle to threshold average

Sample	Average Ct
1/100 Dilution	18.87
1/1000 Dilution	22.418
1/10000 Dilution	25.025

Table 2: PCR solution concentration and corresponding cycle to threshold difference

Sample	Ct Difference
1/100 Dilution	6.155
1/1000 Dilution	2.607

Table 3: PCR solution concentration and corresponding fold change

Sample	Fold Change
1/100 Dilution	71.26
1/1000 Dilution	6.09

In the Excel spreadsheet that is exported from the qPCR software, it has already calculated the average Ct value for each of the dilution samples in Table 1. One can see the relationship between DNA concentration and average Ct, where less concentrated samples result in a

higher Ct value because it takes longer cycles to reach the threshold. The differences of the average Ct were calculated by subtraction, where the 1/10000 dilution sample was selected as the standard to compare the rest of the dilutions to. The resulting values are displayed in Table 2. Using the Ct difference values, the fold change was calculated using the equation, $2^{\text{Ct difference}}$. So, one of the interpretations is that the 1/100 dilution had 71.26 times more DNA amplification than the 1/10000 dilution sample (Table 3).

Discussion

Based on the results, there were discrepancies between the experimental and expected data. For the experimental data, no forward or reverse primers were added into the PCR1 solution and its dilutions due to miscommunication and inadequate supervision during training. Since there were no forward or reverse primers, no amplification was observed for the various samples. To explain the water amplification in Figure 7, there could have been potential contamination to display a change in fluorescence. Due to the inconclusive data obtained from the amplification plot (Figure 7), sample data was used to perform the necessary Ct analysis to determine how much the DNA samples amplified.

In the sample data (Figure 8), there is a proportional relationship between the number of DNA amplified and the fluorescence detected. As DNA amplification increases, fluorescence emission increases. This makes sense as SYBR green dye releases a strong signal whenever it is bound to dsDNA which is formed after DNA amplification. There is no fluorescence signal detected when water is amplified because no dye was added to this sample.

Some limitations to the machine include the cycle threshold that is automatically set. Though one can manually calculate this value during analysis, the machine does not allow for automatic generation of the amplification plot since Ct cannot be customized. While there may be machines for which this is possible, the QuantStudio 5 is made to be autogenerative of threshold and Ct values, so one would need to comb through lots of data to manually set the threshold and Ct average values. This is time consuming, and would be resolved by having an automated process that could be adjusted instead of the all-or-nothing calculations it currently performs.

In addition, the number of samples that can be run is limited by the number of wells in the machine. One could use a probe to multiplex the assays, however this option can be costly, time consuming, and not ideal.

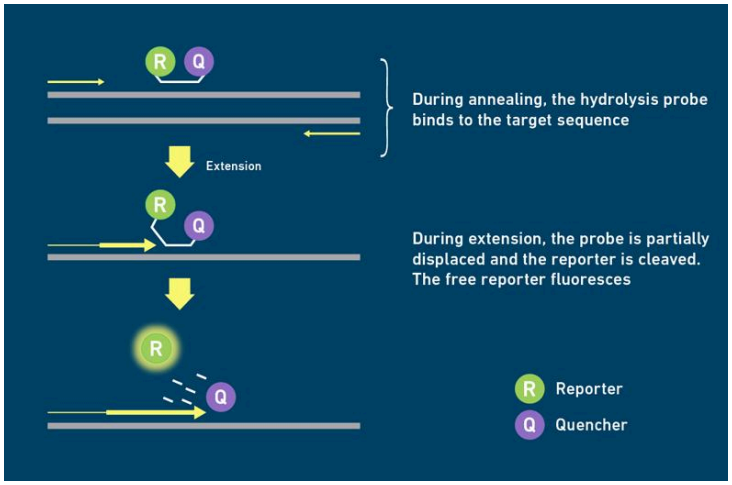


Figure 9: Example of using hydrolysis probe where a specific polymerase cleaves the probe away from the quencher, allowing for its emittance of the fluorescent signal during amplification.

As shown in Figure 9, the probe includes a quencher molecule that is only released at the presence of a specific target sequence. By using various fluorophores, different fluorescent light is emitted which allows for multiplexing of the probes. This means multiple assays can be run in the same well given the machine includes the necessary filters to detect these wavelengths.

The experiment was well-suited for identifying the basic functions of the device. It allowed for identification of trends using dyes such as the proportionality of the quantity of DNA amplified to the fluorescence emitted as well as the increasing concentration of the target which decreases fold change. However, to observe the full functionality of the device, a probe should be used in place of the SYBR green dye for improved assay specificity. In addition, having a treated (could be treated with drugs, environmental conditions, etc) and untreated samples allow for calculation of ΔΔCt which allows for the calculation of relative fold gene expression level as explained in the introduction.

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