1. Introduction

1.1. Overview: The qPCR (Quantitative Polymerase Chain Reaction) service at the Plant-Microbe Genomics Facility utilizes the CFX96 Real-Time PCR Detection system from Bio-Rad, Inc. The system utilizes a 96-well thermal cycler with attached optics to measure fluorescence in each well and proprietary software to interpret the resulting signal. The system can accurately assay thermal-stable DNA polymerase activity in real-time by measuring product formation, and thereby determine the starting concentration of the target DNA. The CFX96 system works by determining the critical threshold cycle, which is the first cycle in which fluorescence is determined to be above background by statistical significance. The critical threshold cycle is inversely correlated to the starting concentration of target DNA. In addition, the system can be used for multiplex reactions, by measuring up to 5 different dyes (and therefore 5 different DNA templates) simultaneously in each well. Flexibility is a key feature that allows for all of the current fluorescence detection methods to be utilized.

1.2. Pre-experimental considerations: Prior to the Facility starting any qPCR work, we request two things: (1) a free face-to-face consultation and (2) a completed qPCR Order form. This communication is necessary to clarify the customer’s goals. Also, qPCR is an exacting technique in so far as it requires precision and uniformity to maximize results. In order for the Facility to best serve the customer, it is essential that the customer have a clear understanding of the qPCR service and the input required. The following are guidelines and recommendations provided to maximize the value of real-time PCR. The guidelines have been provided for the benefit of the customer; they are, of course, not strict rules.

2. Experimental Design

2.1. Selection of Targets: The target, or template, DNA can be genomic DNA, cDNA, mRNA or any other species of DNA that needs to be accurately measured. A standard curve is necessary to determine absolute concentrations of target DNA, and in general is useful to determine the efficiency of a given PCR reaction. It can also be useful for troubleshooting and comparisons. Typically the standard curve is derived
from the target DNA that has been cloned into a plasmid and subsequently highly purified, but any source of DNA that is pure and has an accurately measured concentration could be used for the standard curve. When measuring transcript levels of your gene(s) of interest assaying a reference gene is strongly recommended to control for the variations in quality and quantity of target mRNA between experimental and control samples. For example, beta-actin and glyceraldehyde-phosphate dehydrogenase are some commonly used reference genes.

2.2. Selection of Fluorescence Signal: The signal can be derived from one of two main methods: intercalating dye or a probe (an oligonucleotide with covalently attached dye(s)).

2.2.1. Intercalating Dye: The intercalating dye SYBR Green can be used because it fluoresces 50-100 times greater in the presence of double stranded DNA that results from the extension step of each cycle. Therefore, the fluorescence is proportional to the amount of product formed.

a) Advantages: least expensive; requires least optimization
b) Disadvantages: least sensitive; non-specific; cannot multiplex

2.2.2. Probes: The probes can be of five different varieties (see below). All probes rely on the close proximity of a quenching dye to the reporter dye. In most cases disruption of this quenching interaction causes an increase in fluorescence that is proportional to product formation. However, FRET probes rely on the formation of a quenching interaction and so quantification relies on measuring a decrease in fluorescent signal. As compared to SYBR Green, all probes are more sensitive, and have lower background, but they are more expensive and require greater optimization.

a) Hydrolysis (TaqMan) probes (Fig 1A): These probes are entirely complementary to the target sequence. They have a dye at each end of the oligonucleotide and do not fluoresce when intact. After they bind to the target DNA and are subsequently hydrolyzed by the DNA polymerase, the dyes become separated and fluoresce.
   1. Advantages: easiest to design
   2. Disadvantages: lower sensitivity, higher background

b) Molecular Beacon probes (Fig 1B): These probes have an internal sequence that is complementary to the target DNA. There are also an additional 5-6 bases at each end that self anneal. When the probe binds to the target it fluoresces, but not when it self-anneals.
   1. Advantages: lower background, greater specificity
   2. Disadvantages: more difficult to design and optimize

c) Cleavable Beacons (Fig 1C): These probes are a hybrid between the TaqMan probe and Molecular Beacon. The sequence in the 5’ self-annealing region also is complementary to the target sequence, so the DNA polymerase will hydrolyze this probe similar to a Hydrolysis probe. The dye is efficiently quenched when there is no PCR product, but is also efficiently released when product is present.
1. Advantages: lower background, greater specificity
2. Disadvantages: difficult to design and optimize

d) **Frequency Resonance Energy Transfer (FRET)** probes (Fig 1D): These probes are similar to Hydrolysis probes in that they bind to the target DNA and are hydrolyzed. But there are two probes with one dye, each arranged such that the dyes are adjacent when the two probes bind to the target DNA.
   1. Advantages: highest specificity
   2. Disadvantages: most difficult to design and optimize

e) **Amplifluor Uniprimer** system: This system incorporates a unique sequence into one of the target specific primers thereby creating a binding site for the Uniprimer. The Uniprimer behaves similarly to a molecular beacon in that it binds to itself when it is not incorporated into the amplified product. After incorporation into the PCR product the dyes are separated and therefore the Uniprimer fluoresces. Please see the [Millipore Company website](http://www.millipore.com) for additional details.
   1. Advantages: probe already designed, easily optimized, lower background
   2. Disadvantages: lower sensitivity, can not be multiplexed
Figure 1. Mechanisms by which Real-time PCR probes function. Octagon and yellow star = reporter dye; cross = quenching dye; P = DNA polymerase. mrz, 2001
3. Primer and Probe Design

3.1. References for the Design of Primer Set: Please refer to the Bio-Rad documents listed below for guidelines to design the amplicon primers as well as the fluorescently labeled probe:

3.1.1. Real-Time PCR: General Considerations

3.1.2. Guide to designing primers and probes: Hydrolysis and Molecular Beacon

3.2. Brief Guide to designing primers: The suggestions below are for the PCR primers, whether detection is by SYBR Green, Hydrolysis Probe, or Molecular Beacon. The primers should be 18-25 nucleotides long, and have the same T_m (about 58 to 60°C). The primers should have no more than 2 Gs or Cs among the last five nucleotides at the 3’ end. The G/C content should be about 50 to 60%. The amplified PCR product should be 75 to 150 bases for SYBR green and Hydrolysis probes, but 300 to 400 bases for Molecular Beacons. In order to reduce amplification from contaminating DNA, try to have one or both primers span a known intron/exon junction. To reduce non-specific binding, try designing the primer(s) to bind to untranslated regions, where nucleotide identity is often lower.

3.3. Hydrolysis Probe Design: The probe should be about 20 to 25 bases long, and have a T_m about 8 to 10°C higher than the primers. The probe must not have a G at the 5’ end.

3.4. Molecular Beacon Design: The target or complementary region should be designed the same as a Hydrolysis probe. The self-annealing regions at either end should be 5-6 bases long, palindromic, non-binding to the template, and high in G/C content.

3.5. Cleavable Beacon Design: The design is the same as the Molecular Beacon, except that the 5’ self-annealing sequence is also complementary to the target sequence.

4. Experimental Procedure

4.1. Sample Preparation: Due to the sensitivity of this technology, it is important to take extra measures in sample preparation. If comparative analyses are desired, it is essential that variables between samples are limited to controlled experimental modifications. For this reason, all samples that will be used for comparative analysis should be prepared in an identical manner. This is a PCR based assay, so standard PCR precautions should be used such as the use of clean work areas, gloves, PCR grade water, and filtered tips. In addition, the more accurately the template nucleic acid concentration is measured with absorbance at 260nm or a fluorescent dye, the easier it will be to standardize the results.

4.2. Confirmation of PCR Product: PCR is notorious for amplifying non-specific or unintended targets. Due to this, confirm that the PCR product is in fact the intended target, even if there is only one product, by directly sequencing the PCR product, or cloning the product into a plasmid followed by DNA sequencing. The Plant-Microbe
Genomics Facility can do the DNA sequencing reactions. Confirmation should be done prior to starting the real-time experiments with the CFX96 system.

4.3. Set up of Reaction Mixtures: It is extremely important to minimize variability between wells.

4.3.1. Always use good pipetting techniques.
   a) Use calibrated pipettes, and use the same pipettes across studies as much as possible.
   b) Touching the tip to the well or tube wall after dispensing to remove residual solution.
   c) Aspirate and dispense with a slow, constant rate.
   d) Change tips regularly.
   e) Use sterile, filtered, barrier pipette tips whenever possible.
   f) Set up Negative Control reactions first and seal wells before adding positive controls or unknowns to the plate to minimize cross-contamination.
   g) After adding negative controls and unknowns, cover these wells and add positive controls and standard curve samples to the plate last to minimize cross-contamination.

4.3.2. Make the various solutions in larger quantities and higher concentrations followed by aliquoting and diluting the solution in order to minimize variation between wells.

4.3.3. Aliquoting master mixes to unused columns or rows of 96-well plates or to 8- or 12-tube strips followed by use of a calibrated multi-channel pipette is highly recommended.

4.4. Optimization of Cycling Parameters: The Tₘ of the probes and primers as well as the optimal temperature for the thermostable polymerase determines the cycling parameters. The temperatures and duration times may need to be adjusted slightly if additional products are a problem. You can optimize the conditions with your own thermal cycler, but the greater the differences between your thermal cycler and the CFX96 the more likely you will need to re-optimize with the CFX96 system. The CFX96 system uses a 96-well block with Peltier heating/cooling and a heated lid. If you are not using a Bio-Rad CFX96, then we suggest performing one reaction on the CFX96 at the Facility to determine if the results are the same as for your thermal cycler.

4.5. Control Reactions: There should be wells that lack template in order to detect any contamination and/or primer dimer formation when using SYBR Green. All experiments that utilize reverse transcriptase should have wells or RNA extracts not treated with this enzyme to detect contaminating DNA. The positive control can either be the standard curve reactions, or simply a duplicate of one of the reactions that is known to work well.
4.6. **Standard Curves:** The standard curve should consist of four to six different concentrations ranging between 2 and 10 fold (table 1) differences. Experimental samples should be diluted to a concentration that falls toward the middle of the range of the standard curve; several dilutions may be necessary. Each concentration should be done in duplicate or triplicate.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Starting Quantity DNA Copies/reaction</th>
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<tbody>
<tr>
<td>1</td>
<td>$10^9$</td>
</tr>
<tr>
<td>2</td>
<td>$10^8$</td>
</tr>
<tr>
<td>3</td>
<td>$10^7$</td>
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</tr>
<tr>
<td>7</td>
<td>$10^3$</td>
</tr>
<tr>
<td>8</td>
<td>$10^2$</td>
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</table>

*Table 1:* Suggested ranges of concentration for standard curve. All reactions should be done in duplicate or triplicate.

4.7. **SYBR Green:** For SYBR Green dye the use of a kit is encouraged, since the kits contain dye, enzyme, salts, buffer and nucleotides optimized for real-time PCR. We recommend the Bio-Rad kit iQ SYBR Green Supermix. Kits available from Qiagen, Applied Biosystems, and KAPA Biosystems have also been used successfully in the Facility. In addition, you can use any PCR kit and simply add the SYBR Green dye. The dye should be used at a final dilution of 75,000 to 150,000 times in the reaction well, although these are not absolute limits. Diluting to 125,000 times is a good starting dilution, but in general use as low as concentration as possible since SYBR Green can interfere with the reaction at higher concentrations. As for concentrations of other solutions, simply use your existing PCR concentrations and add the SYBR Green to the Master Mix. The MgCl$_2$ may need to be raised to 5 mM and primers raised to 500 nM.

4.8. **Reaction Volume:** The total volume for each reaction can be between 10 and 50 μL. For beginners, start at the maximum volume recommended by the protocol being used, reducing the volume of each reaction after becoming comfortable with your technique and results in order to reduce costs.

4.9. **Plate and Film:** The Facility will provide the 96-well plates and optically clear film that is required for the CFX96 for no additional cost. The Facility will vortex and centrifuge each reaction plate (if applicable) prior to beginning the reactions.

4.10. **Location for Setting Up reactions:** Clients are welcome to set up their reactions in the Facility, as the Facility has a clean area and all of the necessary bench supplies (charges will be accrued for consumables such as pipette tips). Alternatively, you may obtain a 96-well plate ahead of time, set up the reaction in your lab, and then bring the completed plate to the Facility for analysis. It is highly recommended to set
up your reactions in a clean area, such as a dead-air box, that is strictly reserved for pre-PCR work. Always work in the direction from cleanest to dirtiest, as qPCR is extremely sensitive to contamination.

5. Data analysis

5.1. **CFX Manager Software**: All initial consultations include a copy of the software used to control the CFX thermal cycler and analyze qPCR results. Initial consultations will include instruction on installing and updating the software, as well as creating your thermal cycling protocol and plate maps. One excellent advantage of the CFX Manager Software is the ability to enter all plate map information, as well as standard curves, control wells, fluorophore types, etc., after the data has already been collected. This can be extremely useful when time is a factor, enabling the user to dictate a thermal cycling protocol, collect all data, and analyze at their convenience. Data files from the CFX96 are automatically emailed to the customer upon completion of the data collection.

5.2. **MIQE Guidelines**: The Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines are a set of recommendations published in 2009 by an international group of researchers, with the goal of improving the reporting of qPCR and RT-qPCR experiments. PMGF highly recommends following the MIQE Guidelines to the best of your ability.


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