

Procedures and Recommendations for Quantitative PCR

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1) Introduction

- a) *Overview:* The Quantitative PCR (Polymerase Chain Reaction) section of the Plant-Microbe Genomics Facility utilizes the iCycler iQ Real-Time 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 one of the substrates, *i.e.* the target DNA. The iQ 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, *i.e.* measure up to 4 different dyes simultaneously in each well and therefore 4 different DNA templates. Flexibility is a key feature that allows for all of the current fluorescence detection methods to be utilized.
- b) *Pre-experimental considerations:* Prior to the Facility starting any Quantitative PCR work, we request two things: (1) a free face-to-face consultation and (2) a completed Quantitative PCR Order form. This communication is necessary to clarify the customer's goals. Also, quantitative PCR 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 quantitative PCR 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

- a) *Selection of Targets:* The target, *i.e.* 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, *i.e.* 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.

- b) *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)).
- i) *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.
- (1) Advantages: least expensive; requires least optimization
 - (2) Disadvantages: least sensitive; non-specific; can not multiplex
- ii) *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.
- (1) Hydrolyzable probes (Fig 1A): *i.e.* Taqman probes, are entirely complementary to the target sequence. These probes have a dye at each end of the oligonucleotide and do not fluoresce when intact. But after they bind to the target DNA and are subsequently hydrolyzed by the DNA polymerase the dyes become separated and fluoresce.
 - (a) Advantages: easiest to design
 - (b) Disadvantages: lower sensitivity, higher background
 - (2) Displaceable probes (Fig 1B): *i.e.* Molecular Beacons, have internal sequence that is complementary to the target similar to the Taqman probe but in addition 5-6 bases at each end that self anneal. When the probe binds to the target it fluoresces, but not when it self-anneals.
 - (a) Advantages: lower background, greater specificity
 - (b) Disadvantages: more difficult to design and optimize
 - (3) Cleavable Beacons (Fig 1C): 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 Taqman probe. The dye is efficiently quenched when there is no PCR product, but is also efficiently released when product is present.
 - (a) Advantages: lower background, greater specificity
 - (b) Disadvantages: difficult to design and optimize
 - (4) Frequency Resonance Energy Transfer (FRET) probes (Fig 1D): are similar to Taqman 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.
 - (a) Advantages: highest specificity
 - (b) Disadvantages: most difficult to design and optimize
 - (5) Amplifluor Uniprimer system: incorporates a unique sequence into one of the target specific primers thereby creating a binding site for the Uniprimer, *i.e.* probe, that 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 Intergen Company website for additional details:

http://www.intergen.com/body_pcr_amplifluor.html.

- (a) Advantages: probe already designed, easily optimized, lower background
- (b) Disadvantages: lower sensitivity, can not be multiplexed

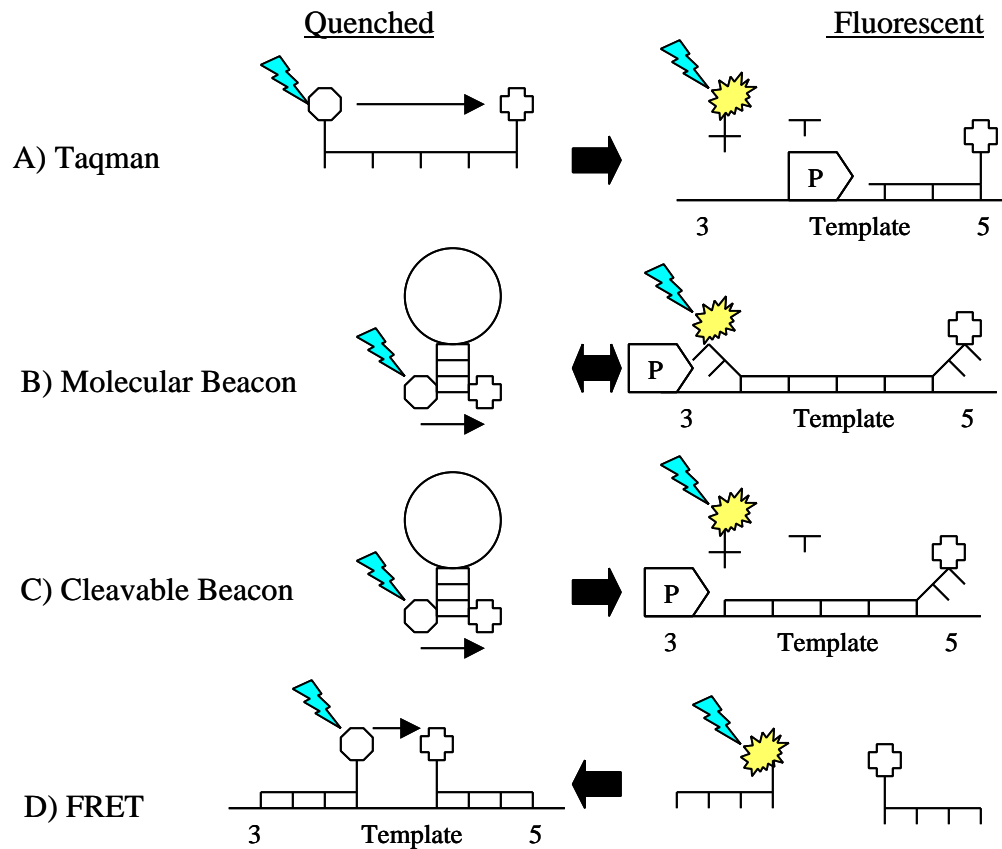


Figure 1. Mechanisms by which Real-time PCR probes function. octagon and yellow star = reporter dye; cross = quenching dye; P = DNA polymerase.

3) Primer and Probe Design

- a) *References for the Design of Primer Set:* Please refer to the BioRad documents listed below for guidelines to design the amplicon primers as well as the fluorescently labeled probe:
 - i) Real-Time PCR: General Considerations
(<http://www.biosci.ohio-state.edu/~pmgf/BioRad-QPCRgeneral.pdf>)
 - ii) Guide to designing primers and probes: Taqman and Molecular Beacon
(http://www.biosci.ohio-state.edu/~pmgf/probe&primer_design.htm)
- b) *Brief Guide to designing primers:* The suggestions below are for the primers whether detection is by SYBR Green, Taqman, 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 amplicon, i.e. the amplified PCR product, should be 75 to 150 bases for SYBR green and Taqman probes, but 300 to 400 bases for Molecular Beacons. In order to reduce amplification from contaminating DNA try having one or both primers span a known intron/exon junction. To reduce non-specific binding try having the primer(s) bind to untranslated regions where nucleotide identity is often lower.

- c) *Taqman 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.
- d) *Molecular Beacon Design:* The target or complementary region should be designed the same as a Taqman 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.
- e) *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

- a) *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 standardize the results.
- b) *Confirmation of PCR Product:* PCR is notorious for amplifying non-specific or unintended targets so the product of the primers should be confirmed to be indeed the intended target even if there is only one product. Confirmation of the product should be done 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 iQ system.
- c) *Set up of Reaction Mixtures:* It is important to minimize variability between wells since variations during pipeting can be detected. Therefore always use good pipeting techniques, such as touching the tip afterwards to remove residual solution, aspirating and dispensing with a slow, constant rate, changing tips regularly, etc. Make the various solutions in larger quantities and higher concentrations followed by aliquoting and diluting the solution in order to minimize variation between wells. Table 1 is a real example of how the experimental solutions were set up to minimize variation as well as provide sufficient volume for each subsequent step.

Step 1: Make Master Mix - 125µl SYBR Green Buffer, 10x
 150µl MgCl₂, 25mM
 100µl dNTP blend, 2.5mM each
 6.25µl Amplitaq Gold, 5U/µl
 381.25µl Total Volume

Step 2:	Tube	Type	Master Mix(µl)	Primers(µl)		pGEM (µl)	Water (µl)	Total Vol(µl)
				F	R			
	1	background	61.0	-	-	4	135.0	200
	2	neg control	61.0	10	10	-	119.0	200
	3	10 ⁴ pg pGEM	61.0	10	10	4	115.0	200
	4	10 ³ pg pGEM	61.0	10	10	4	115.0	200
	5	10 ² pg pGEM	61.0	10	10	4	115.0	200
	6	10 ¹ pg pGEM	61.0	10	10	4	115.0	200

Step 3: From each tube in step 2, place 47µl in each of 4 adjacent wells of the 96-well thermal cycler plate.

Table 1. Procedure for setting up solutions for a test of the Applied Biosystems SYBR Green PCR kit on the BioRad iCycler iQ Real-Time Detection System. Each primer is at a final concentration of 100nM, and the pGEM plasmid was diluted to an appropriate concentration prior to adding it to the above tubes.

- d) *Optimization of Cycling Parameters:* The T_m 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 iCycler the more likely you will need to re-optimize with the iQ system. The iQ system uses a 96-well block with peltier heating/cooling and a heated lid. If you are not using a BioRad iCycler, then I suggest performing one reaction on an iCycler in the Facility to determine if the results are the same as for your thermal cycler.
- e) *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 be the standard curve reactions, or simply do one reaction in duplicate that is known to work well. The standard curve should have four to five different concentrations with changes of 2 fold to 10 fold, e.g. see table 2, for each different concentration. Each concentration should be in duplicate or triplicate.
- f) *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 BioRad kit iQ SYBR Green Supermix. Kits available from Qiagen and Applied Biosystems have also been used successfully in the Facility. In addition you can use any PCR kit, and add SYBR Green dye from Molecular Probes, Inc. 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 125,000 times is a good dilution to start at, 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. **In addition, fluorescein (BioRad P/N 170-8780) is required in all wells in order to generate the Well Factors for all mixes other than the iQ SYBR Green Supermix kit.** These are internal calibration values that the iCycler uses for each well for each reaction. The fluorescein generates sufficient signal for the iCycler to measure fluorescence from the well, but not enough signal to interfere with the reaction. Fluorescein must be at a final concentration of 10 nM in each well.

- g) *Reaction Volume*: The total volume for each reaction can be between 25 and 50 μ l. You should start at the maximum volume, and then possibly reduce the volume after becoming comfortable with your technique and results in order to reduce costs.
- h) *Arrangement of Reactions in Wells*: Place the replicated reactions in wells adjacent to each other in rows, *e. g.* wells A1, A2, A3 would have identical reactions. Arrange all reactions related to a specific gene in adjacent rows. For example, place the standard curve and experimental reactions for your gene of interest in rows A, B and C, then place the same reactions for the house keeping gene in rows D, E, and F. This arrangement will make it easier to program the iCycler and to analyze the results.
- i) *Plate and Film*: The Facility will provide the 96-well plates and optically clear film that is required for the iCycler at cost. The Facility has a centrifuge that is capable of spinning the plates in order to place all solutions at the bottom of the well.
- j) *Location for Setting Up reactions*: You are welcome to set up the reactions in the Facility, but you should bring your own supplies, tips and pipetman. 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.

5) Outline of Steps to Organize Experiment

- a) Select gene of interest, and house keeping gene (if necessary)
- b) Select detection system: Intercalating Dye or Dye-labeled Probe
 - i) If a probe, then what type: Taqman, Molecular Beacon, etc.
- c) Design primer sets and probes (if necessary)
- d) Order primers
- e) Optimize PCR conditions
 - i) If specificity can not be achieved, then redesign and reorder primers
- f) Order probe (if necessary)
- g) Perform the real-time PCR analysis

6) References

- a) General information about real-time PCR:
 - i) Giulietti, A. et al. 2001. An Overview of Real-Time Quantitative PCR: Applications to Quantify Cytokine Gene Expression. *Methods*. 25: 386-401.

- b) Use of iQ/iCycler Real-time PCR system:
 - i) Gavrilin, M. A. et al. 2000. Monocyte Chemotactic Protein 1 Upregulates IL-1B Expression in Human Monocytes. *Biochemical and Biophysical Research Communications*. 227: 37-42.

- c) Data Analysis:
 - i) Muller, P. et al. 2002. Processing of Gene Expression Data Generated by Quantitative Real-Time RT-PCR. *Biotechniques*. 32:1372-1379.