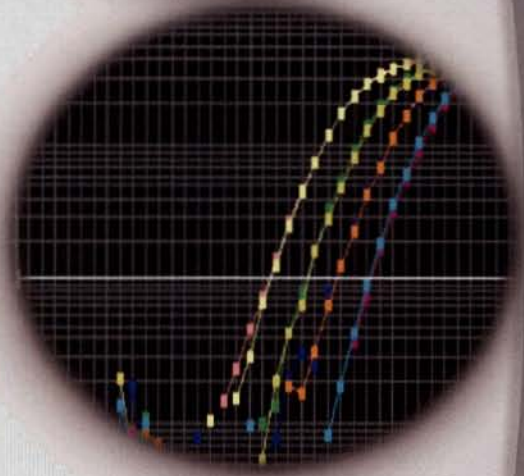


I Curso Nacional
PCR en Tiempo Real

Aplicado a la detección de patógenos en plantas y semillas

Fitopatología de Yuca y Frutas Tropicales
CIAT, Palmira
Agosto 3-6, 2009

Manual de Métodos y Aplicaciones

PCR en Tiempo Real Aplicado a la Detección de Patógenos en Plantas y Semillas, I Curso Nacional

CENTRO INTERNACIONAL DE AGRICULTURA TROPICAL
MINISTERIO DE AGRICULTURA Y DESARROLLO RURAL
COLCIENCIAS

August 3, 2009
Patología de Yuca y Frutas Tropicales – CIAT

CENTRO INTERNACIONAL DE AGRICULTURA TROPICAL, CIAT
KILOMETRO 17 RECTA CALI-PALMIRA
APARTADO AEREO 6713
CALI, COLOMBIA
TELEFONO:(57-2) 445 00 00
FAX: (57-2) 445 00 73
E-MAIL: E.ALVAREZ@CGIAR.ORG
SITIO WEB: WWW.CIAT.CGIAR.ORG

EL CONTENIDO DE ESTA PUBLICACIÓN FUE PREPARADO POR EL PROGRAMA DE PATOLOGÍA DE YUCA Y FRUTAS TROPICALES Y THE UNIVERSITY OF GEORGIA, PLANT PATHOLOGY.

EDICION Y DISEÑO: PROGRAM PATOLOGIA DE YUCA Y FRUTAS TROPICALES, AGOSTO DE 2009, IMPRESO EN COLOMBIA

Tabla de Contenido

PCR en Tiempo Real Aplicado a la Detección de Patógenos en Plantas y Semillas, I Curso Nacional

Introducción general

Los métodos convencionales de identificación de patógenos han dependido generalmente de la identificación de síntomas de la enfermedad, aislamiento y cultivo de los organismos mediante pruebas morfológicas y bioquímicas. Las mayores limitantes de estas estrategias basadas en la morfología de los agentes causantes de la enfermedad, son la capacidad del organismo a ser cultivado, el tiempo requerido y el requerimiento de amplia experiencia taxonómica. Además, el diagnóstico de enfermedades de plantas puede ser aun más difícil cuando se trata de material de propagación vegetativa asintomático tales como material de vivero a ser utilizado en la producción de injertos en árboles frutales o en el caso de tubérculos de papa, estacas de yuca, etc.

El uso de métodos moleculares puede resolver muchas de estas deficiencias. En consecuencia, ha habido avances significativos en el área de la detección molecular de fitopatógenos en las ultimas tres décadas. La llegada de la detección basada en anticuerpos, los anticuerpos monoclonales y la técnica de ELISA, fueron un punto de partida importante en virología y bacteriología. Luego le siguieron las tecnologías basadas en el ADN, tales como la reacción en cadena de la polimerasa (PCR), la cual revolucionó el diagnostico molecular y las ciencias biológicas.

En la última década, el rango de problemas que pueden ser diagnosticados utilizando PCR diagnostico ha crecido enormemente. La alta flexibilidad y variaciones de aplicación específica en el tema básico del sistema han permitido el desarrollo de muchas variantes de la PCR adaptadas a un rango amplio de aplicaciones. Adicionalmente, la PCR diagnostica ha sido mejorada grandemente por medio de la introducción de la PCR de segunda generación, conocida como PCR en tiempo real, en la cual es posible la detección de patógenos por fluorescencia en tubos cerrados y la cuantificación durante la amplificación de la PCR (en tiempo real), eliminando así la necesidad de etapas laboriosas de procesamiento de muestras posteriores a la PCR, lo que reduce grandemente el riego de contaminación.

Con el uso de la PCR en tiempo real es posible, no solo detectar la presencia o ausencia del patógeno problema, sino que también cuantificar éste en la muestra, permitiendo una evaluación cuantitativa del número de propágalos en la misma. La enumeración del patógeno durante la detección es crucial para estimar los riesgos potenciales con respecto al desarrollo de la enfermedad y provee bases útiles para la toma de decisiones en el manejo de la misma.

Los cultivos pueden ser atacados por muchos patógenos, los cuales adicionalmente, ocurren frecuentemente en complejos. Por lo tanto, muchos aplicaciones de diagnostico de enfermedades requieren la detección simultánea y la cuantificación de varios patógenos. Las limitaciones metodológicas, sin embargo, son en muchos casos la razón para desarrollar pruebas diseñadas para pocos patógenos (Simplex). La tecnología de los Microarreglos de ADN, originalmente diseñada para estudiar expresión génica y generar polimorfismo de perfiles de un solo nucleótidos (SNP), actualmente es una tecnología emergente para el diagnostico de patógenos, la cual en teoría, ofrece una plataforma de capacidad múltiple ilimitada (Multiplex). Esta es vista como una tecnología que fundamentalmente crea un cambio en los diagnósticos moleculares.

El crecimiento rápido de la base de datos generada por investigaciones sobre genómica y biosistemática provee oportunidades únicas para el diseño de pruebas moleculares más versátiles, de alto rendimiento, sensibles y especificas las cuales podrían resolver las mayores limitaciones de las tecnologías actuales y beneficiar a la fitopatología.

Sin embargo, mientras la especificidad y sensibilidad en la detección de patógenos se ha mejorado grandemente y la detección de los patógenos se ha hecho más simple y rápida, aun existen algunos problemas de naturaleza técnica y económica, los cuales requieren ser resueltos para asegurar la emergencia de un sistema de detección confiable que sirva para aplicaciones rutinarias.

Este manual presenta una revisión resumida de los principios de la PCR de nueva generación o PCR en tiempo real, sus ventajas, desventajas y aplicaciones en la detección de fitopatógenos tales como virus, fitoplasmas y bacterias. Como ejemplos de caso de estas aplicaciones, se presenta la metodología básica para la detección del virus asociado con la enfermedad del Cuero de Sapo en Yuca (CFSV), la detección del fitoplasma 16SrIII-L también asociado con el cuero de Sapo de la yuca y para la detección de la bacteria *Ralstonia solanacearum*, causante del marchitamiento en plátano. Finalmente, el manual presenta un compilación con más de 150 publicaciones científicas en las cuales se muestran las diferentes aplicaciones de la tecnología para el diagnóstico y detección de fitopatógenos de naturaleza viral, bacteriana, fungosa y fitoplasmas, los cuales se espera aporten bases científicas importantes para los investigadores que trabajan en ésta área y los que deseen incursionar en ella más adelante.

QPCR PRINCIPIOS Y GENERALIDADES

(Source: Introduction to Quantitative PCR: Methods and Applications Guide - Stratagene®)

Experimental design

The core idea that will guide the development of your experimental design is: "What is the fundamental scientific question that you are trying to answer?". For each project, there are a number of considerations that need to be addressed:

- What is the goal of the experiment?
- What questions are to be answered?
- What are the systems being studied?
- What is the total number of genes to be analyzed?
- What control samples (calibrators) and genes (normalizers) will be used to measure the changes in expression levels?
- What is the source of the target sequence?
- Are there any limitations to the amount of target material available?
- What is the sensitivity required to obtain the data necessary to answer the experiem's fundamental question?

The answers to these questions will determine which PCR approach Is best to the requirements and objectives of the experiments (Figure 1).

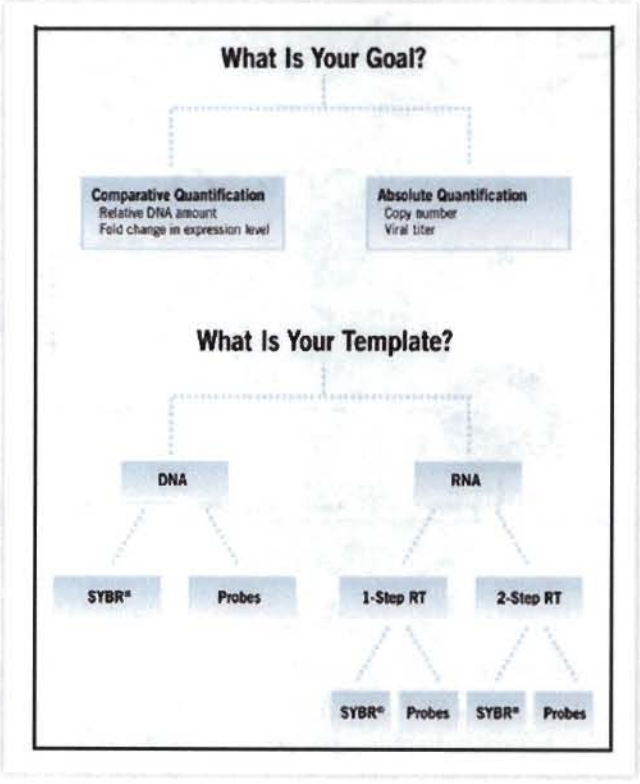


FIGURE 1: INTRODUCCION TO QUANTITATIVE PCR - STRATAGENE®

Figure 1. Flowchart showing a typical experimental design process on the goals and requirements of the assay.

Methods of Quantification

There are two basic quantification methods, and each is suitable for different applications: standard curve and relative quantification.

Standard Curve

The most direct and precise approach for analyzing quantitative data is to use a standard curve that is prepared from a dilution series of template of known concentration. This is known as "standard curve" or "absolute" quantification. The standard curve approach is used when it is important to the experimental design and objective of the project to measure the exact level of template in the samples (e.g., monitoring the viral load in a sample).

A variety of sources can be used as standard templates. Examples include a plasmid containing a cloned gene of interest (GOI), genomic DNA, cDNA, synthetic oligos, in vitro transcripts, or total RNA such as Stratagene's QPCR Human Reference Total RNA.

Figure 2 describes a basic setup for standard curve quantification. Keep in mind the selection of template is dependent upon the application being pursued. The most critical consideration is that the primer set be optimized to work efficiently with the standars and the experimental source material or tissue.

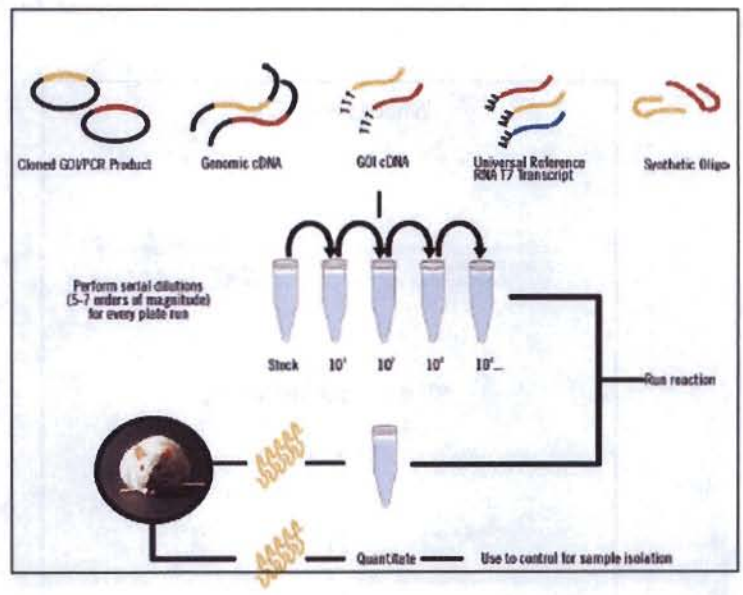


FIGURE 2: INTRODUCCION TO QUANTITATIVE PCR - STRATAGENE®

Figure 2. Experimental setup for standard curve quantification.. Using a known starting concentration of template from one of a variety of sources, a dilution series is performed. These samples are run under the standard well type on the same plate as your unknowns. By comparing the Ct values of the unknowns to the Ct values of the standards, the starting template quantities for the unknown samples can be calculate.

Following amplification of the standard dilution series, the standard curve is generated by plotting the log og the initial template copy number against the Ct generated for each dilution. If the aliquotting was accurate and the efficiency of the amplifications does not change over the range of template concentrations being used, the plot o these points should generate a linear regression line. This line represents the standard curve. Comparing the Ct values of the unknown samples to this standard curve allows the quantification of initial copy numbers (Figure 3)

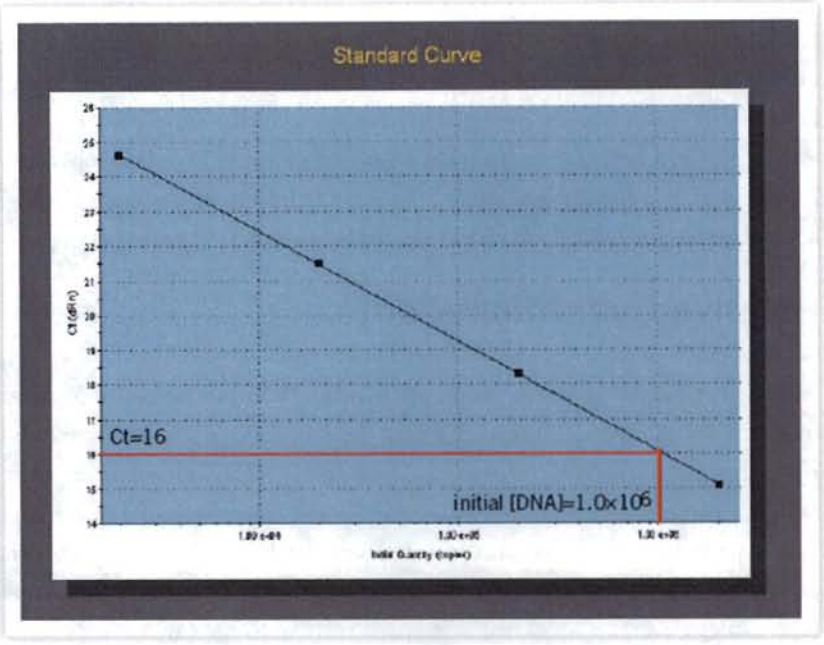


Figure 3. Ilustration of the theory behind standard curve quantification. The log of the initial template quantity is plotted against the Ct values for the standard. By comparing the Ct values of the unknowns to this Standard Curve plot, the initial template quantities for unknown samples can be determined.

Ideally, a standard curve will consist of at least 4 points, and each concentration should be run at least in duplicate (the more points the better). The range of concentrations in the standard curve must cover the entire range of concentrations that will measure in the assay (this may be several orders of magnitude). Conclusions cannot be drawn from samples whose calculated initial quantity exceeds the range of the curve.

In addition, the curve must be linear over the whole concentration range. The linearity is denoted by the R squared (Rsq) value (R2 or Pearson Correlation Coefficient) and should be very close to 1 (≥ 0.985). A linear standard curve also implies that the efficiency of amplification is consistent at varying template concentrations. If the standard curve becomes non-linear at very low template concentration, it is probably approaching he limit of detection for the assay. Unknown samples that have Ct values that fall within a non-linear section of the standard curve cannot be accurately quantified. Ideally, the efficiency of both the standard curve and sample reactions should be between 90 and 110%. One hundred percent efficiency implies perfect doubling of amplicon each cycle. If the efficiency is significantly less, this implies the reaction is being slowed in some way, either from inhibitors present in the reaction mix or suboptimal primer sets or reaction conditions. Efficiencies significantly above 100% typically indicate experimental error (e.g., miscalibrated pipettors, PCR inhibitors, probe degradation, formation of non-specific products, and formation of primer dimmers). Primer dimmer formation is typically of greatest concern with SYBR® Green I assays where any double-stranded product will be detected. Deviation in efficiency can also be due to poor serial dilution preparation as well as extreme ranges of concentrations that either inhibit PCR (high template amounts) or exceed the sensitivity of particular assay (very low amounts). The most important aspect is to have the efficiencies of standards and targets within about 5% of each other if possible, with both near 100%.

Once the reactions for the standard curve and the samples have been optimized, Ct values can be compared to each other and an initial template quantity can be estimated. It is important to remember that for this type of quantification a standard curve must be run on the same plate as the known samples.

Replicates can vary in Ct when run at different times or on different plates, and thus are not directly comparable to other runs. Also keep in mind that the "absolute" quantity obtained from the standard curve is only as good as the DNA/RNA quantification methods used to measure the standards, so you must take care to use very clean template and to perform replicate measurements (whether using UV spectrophotometry or nucleic acid binding dyes such as RiboGreen® and PicoGreen® dyes). At least 2-3 no template control (NTC) wells and 2-3 no reverse transcriptase control wells (for QRT-PCR) runs) should be included. Standard curve analysis is explained in later sections.

Relative Quantification

Although standard curve (or absolute) quantification can be useful in determining absolute quantities of target, the majority of scientific questions regarding gene expression can be accurately and reproducible answered by measuring the relative concentration of the gene of interest (GOI) in unknown samples compared to a calibrator, or control sample. Here, the calibrator is a baseline for the expression of a given target gene. This can be zero time point in a time-course experiment or an untreated sample that will serve as a benchmark to which the other samples can be compared. Using this approach, differences in Ct value between an unknown sample and calibrator are expressed as fold-changes (i.e., up- or down-regulated) relative to the calibrator sample. In addition to comparing the expression of the target gene alone in a control versus experimental sample, it is always a good idea to normalize the results with a normalizing reference gene or target, typically a gene whose expression is constant in both the control (calibrator) and experimental samples. This normalization controls for differences in RNA isolation and the efficiency of the reverse transcription reaction from sample to sample and experiment to experiment. Normalizers are explained in more detail in later sections.

When designing a comparative quantification experiment, it is not necessary to run a standard curve on every plate as you would for absolute quantification. Rather the results are expressed as the fold difference between the target and normalizer in experimental versus calibrator samples. However, it is usually not accurate to assume that the amplification efficiency in any reaction is going to be 100%, or that the same concentrations of template molecules will be detected at a given Ct value each time the assay is run. Actual amplification efficiency values for a particular reaction can be established via a standard curve measurement during assay design, and multiple standard curves should be run to verify that this efficiency measurement is reproducible (typical run-to-run variability is in the 5% range).

QPCR Chemistry Options

The fluorescent reporter molecule used in real-time PCR reactions can be (1) a sequence specific probe comprised of an oligonucleotide labelled with a florescent dye plus a quencher [e.g., TagMan® probes (hydrolysis probes), Molecular Beacons and Scorpions] or (2) a non-specific DNA binding dye such as SYBR Green I that fluoresces when bound to double-stranded DNA. The criteria that should be used to select the chemistry for your QPCR experiment are based on the following considerations:

- The level of sensitivity and accuracy required for the data analysis
- The budget available to support the project
- The skill and experience of the researcher in designing and optimizing QPCR assays.
- The number of targets to be analyzed and whether a multiplex assay is appropriate

The overall objective and requirements of the research project must be considered in deciding on the most appropriate detection chemistry. This guide will cover the design of experiments using the most commonly utilized chemistries: SYBR Green I DNA binding dye, Taq Man probes, Molecular Beacons, and Scorpion probes. Each QPCR chemistry option has advantages and drawbacks. Chemistries such as Amplifluor® primers, LUX primers, FRET probe pairs (also known as hybridization probes), or Invader® probes will work in the Mx system, however they are beyond the scope of this application guide.

DNA Binding Dyes

DNA binding dyes such as SYBR Green I are cost effective and easy to use, especially for researchers who are new to QPCR techniques. These factors make SYBR Green I a common choice for optimizing QPCR reactions.

When free in solution, SYBR Green I displays relatively low fluorescence, but when bound to doubles-stranded DNA its fluorescence increases by over 1000-fold. The more doubled-stranded DNA that is present, the more binding sites are for the dye, so florescence increases proportionately to DNA concentration. This property of the dye provides the mechanism that allows it to be used to track the accumulation of PCR product. As the target is amplified, the increasing concentration of double-stranded DNA in the solution can be directly measured by the increase in fluorescence signal (Figure 4). Compared to probe-based methods, SYBR Green I assays are relatively easy to design and run. All that is necessary is to design a set of primers,, optimize the amplification efficiency and specificity, and the run the PCR reaction in the presence of the dye.

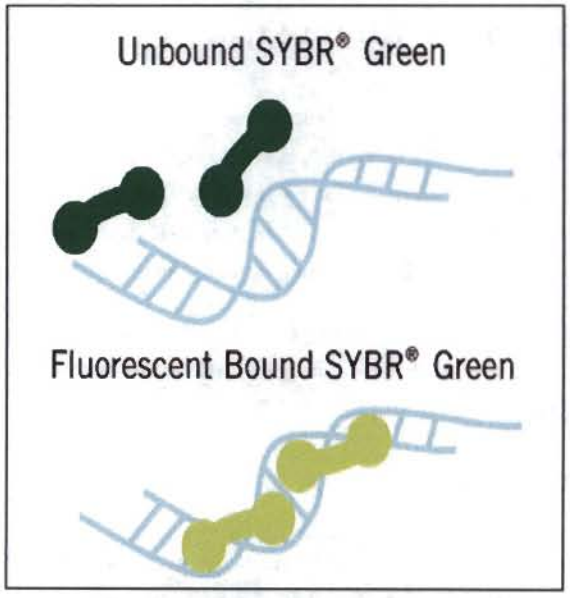


Figure 4. SYBR® Green I detection mechanism: double-stranded DNA in the reaction is bound by yhe dye, SYBR® Green I is 1000-fold more fluorescent than in the unbound state. As PCR amplification increases the amount of dsDNA present, the fluorescence signal increases proportionally.

FIGURE 4: INTRODUCCION TO QUANTITATIVE PCR - STRATAGENE®

One limitation of assays based on DNA-binding dye chemistry is the inherent non-specificity. SYBR Green I will increase in fluorescence when bound to any double-stranded DNA. Therefore the reaction specificity is determined solely by the primer.

Consequently, the primers should be designed to avoid non-specific binding (e.g., primer dimmer formation). Otherwise, it is possible that the measured fluorescence may include signal contamination resulting in artificially early Ct values, given an inaccurate representation of the true target concentration. A non-specific signal cannot always be prevented, but its presence can be easily and reliably detected by performing melting curve analysis on the PCR products from every run.

Following the amplification reaction, the PCR products can be slowly melted while the SYBR Green I fluorescence is detected. As the temperature increases, the DNA melts and the fluorescence intensity decreases. The temperature at which a DNA molecule melts depends on its length and sequence; therefore, if the PCR product consist of molecules of homogeneous length and sequence, a single thermal transition will be detected. On the other hand, the presence the more than one population of PCR products will be reflected as multiple thermal transitions in the fluorescence intensity. In this way, the fluorescence versus temperature curve (also known as the dissociation curve) is used to differentiate between specific and non-specific amplicons based on the Tm (melting temperature) of the reaction end-products.

DNA binding dyes are often used to initial expression validation screening of microarray samples as well as for other gene expression applications not requiring exceptional specificity. Optimization of primers to use with SYBR Green I chemistry is straightforward and provides a high level of QPCR experimental design success.

Probe- Based Chemistries

As compared to non-specific chemistries such as SYBR Green I dye, a higher level of detection specificity is provided by using an internal probe to detect the QPCR product of interest. In the absence of a specific target sequence in the reaction, the fluorescent probe is not hybridized, remains quenched, and does not fluoresce. When the probe hybridizes to the target sequence of interest, the reporter dye is no loger quenched, and fluorescence will be detected. The level of fluorescence detected is directly related to the amount of amplified target in each PCR cycle.

A significant advantage of using probe-based chemistry compared to using DNA binding dyes is that multiple probes can be labelled with different reporter dyes and combined to allow detection of more than one target in a single reaction, referred as multiplex QPCR.

Linear Probes

Linear probes (i.e., hydrolysis or TaqMan probes) are the most widely used and published detection chemistry for QPCR applications. In addition to the PCR primers, this chemistry includes a third oligonucleotide in the reaction known as the probe. A fluorescent reporter dye, typically FAM™, is attached to the 5′end of the probe and a quencher, historically TAMRA™, is attached at the 3′end. Increasingly, dark quenchers such as Black Hole Quenchers® (BHQ) are replacing the use of TAMRA because they provide lower background fluorescence. As long as the two molecules (reporter and quencher) are maintained in close proximity, the fluorescence from the reporter is quenched and no fluorescence is detected at the reporter dye's emission wavelength. TaqMan probes use a FRET (Fluorescence Resonance Energy Transfer) quenching mechanism where quenching can occur over a fairly long

distance (100Å o more, depending on the fluorophore and quencher used), so that as long as the quencher is on the same oligonucleotide as the fluorophore, quenching will occur.

The probe designated to anneal to one stand of the target sequence just slightly downstream of one of the primers. As the polymerase extends that primer, it will encounter the 5'end of the probe. *Taq* DNA polymerase has 5'-3'nuclease activity, so when *Taq* DNA polymerase encounters the probe it displaces and degrades the 5'end, releasing free reporter dye into solution. Following the separation of reporter dye and quencher, fluorescence can be detected from the reporter dye (Figure 5).

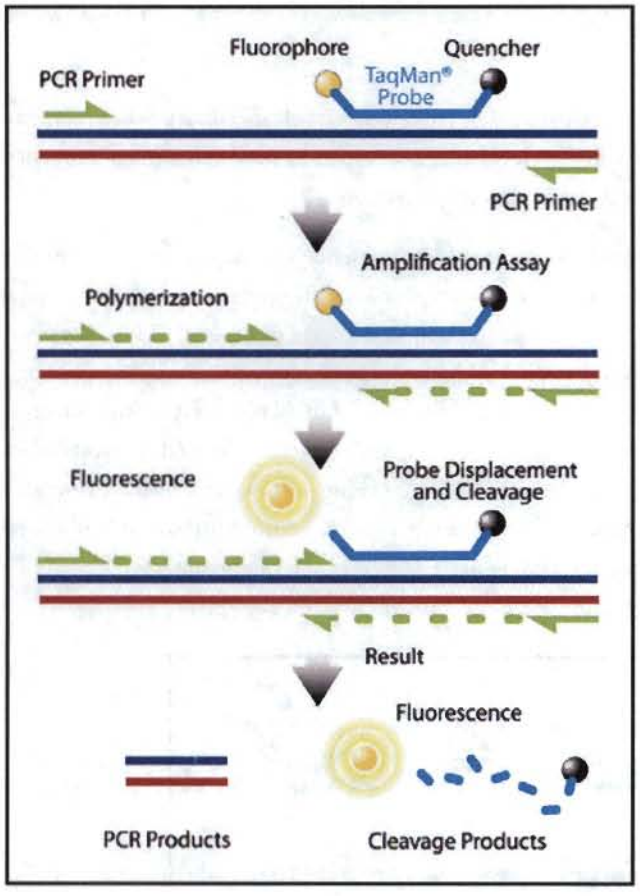


FIGURE 5: INTRODUCCION TO QUANTITATIVE PCR - STRATAGENE®

Figure 5. TaqMan® probe chemistry mechanism. These probes rely on the 5'-3'nuclease activity of *Taq* DNA polymerase to cleave a dual-labelled probe during hybridization to the complementary target sequence.

In order to optimize probe binding and subsequent cleavage, it is critical to adjust the thermal profile to facilitate both the hybridization of probe and primers, and the cleavage of the probe. To meet both of these requirements, linear probes will generally use two-step thermal profile with a denaturing step (usually at 95°C) and a combination annealing-extension step at 60°C, 7-10°C below the Tm of the probe. If the temperature in the reaction is too high when *Taq* DNA polymerase extends through the primer (such as at a standard extension temperature of 72°C) the probe will be strand-displaced rather tan cleaved and no increase in fluorescence will be seen.

TaqMan chemistry can be used for SNP detection or mutation analysis in a multiplex reaction where a separate probe is designed for each allele and each probe is labelled with a different fluorophore (e.g., with FAM and HEX™). Each

probe is designed so that is complementary to one allele sequence and not the other. However, in these assays, it can be challenging to optimize conditions to prevent the probes from annealing non-specifically to the wrong allele. In general, enhanced specificity for SNP and allele discrimination analysis is achieved by using either one of the structure probe chemistries described in the "Structure Probes" section (below) or with a new type of TaqMan probe known as a Minor Groove Binder (MGB) TaqMan probe. The MGB probes are similar to the standard TaqMan probes, but they include the addition of a minor groove-binding moiety on the 3'end that acts to stabilize annealing to the template. The stabilizing effect that the MGB group has on the Tm of the probe allows for the use a much shorter probe (down to ~13 bp). The shorter probe sequence is more susceptible to the destabilizing effects of single mismatches, which makes these probes better than standard TaqMan probes for applications that require discrimination of targets with high sequence homology.

Structures Probes

Structured probes contain stem-loop structure regions that confer enhanced target specificity when compared to traditional linear probes. This characteristic enables a higher level of discrimination between similar sequences and makes these chemistries well suited for SNR and allele discrimination applications.

Molecular Beacons include hairpin loop structure, where the central loop sequence is complementary to each other. One end (typically 5') of the stem is modified with a reporter fluorophore and the other end carries a quencher. Rather than using a FRET-quencing mechanism similar to TaqMan probes, Molecular Beacons rely on ground-state or static quenching, which requires the fluorophore and quencher to be in very close proximity for quenching to occur. Historically, DABCYL or Mehyl Red has been used for this application, but BHQs are becoming increasingly common. In the absence of target sequence, the stem loop structure is energetically favored and this places the fluorophore and quencher immediately adjacent to one another so that quenching will occur. In the presence of the target sequence, the annealing of the loop sequence to the target is the preferred conformation. When annealed to the target, the fluorophore and quencher are separated, and the reporter fluorescence can be detected (Figure 6).

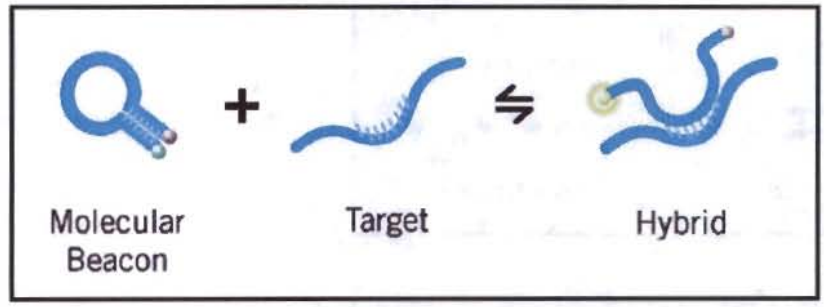


Figure 6. Molecular Beacon chemistry mechanism. The Molecular Beacon includes a hairpin-loop structure, with the loop complementary to a target sequence and the stem formed by the addition of internal complementary sequences. When hybridized to the target, the fluorophore and quencher are far enough apart to allow fluorescence to be detected.

In the absence of the specific target, the Molecular Beacon's thermodynamic properties favour the formation of the hairpin over mismatched binding. This property gives Molecular Beacons the increased mismatch discrimination that makes then well suited for applications such as SNP detection and allele discrimination.

Since the Molecular Beacon chemistry does not rely on the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase, it can be used in a traditional three-step thermal profile. When the thermal cycling ramps up to 72°C and the Taq DNA polymerase extends to where the Molecular Beacon probe is annealed, the probe will simply be displaced and it will assume the hairpin loop conformation again. Because formation of the Molecular Beacon hairpin loop is a reverse process, the probe will be recycle with each PCR cycle.

Careful design of the Molecular Beacon stem is critical to ensure optimized performance of the reaction. If the stem structure is too stable, target hybridization can be inhibited. In addition, if the Molecular Beacon probe does not fold in the expected stem loop conformation, it will not quench properly. Any Molecular Beacon probe should be tested after synthesis to verify that it is behaving as expected before it is used in any QPCR assays. Melt curves can be used to make this determination (Figure 7). By melting the Molecular Beacon alone, in the presence of its perfect complement, or a mismatched sequence, the dynamics of the reaction can be easily compared and used to determine the optimal temperature for fluorescence measurement and mismatch discrimination.

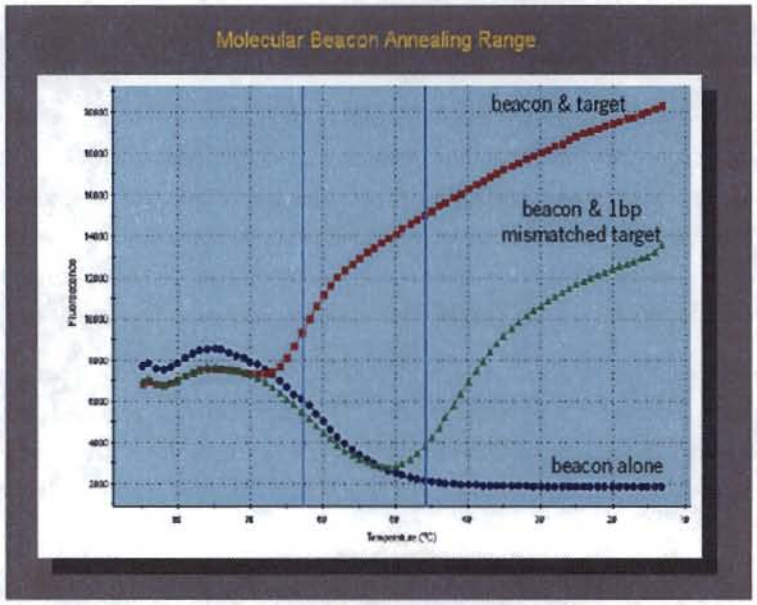


Figure 7. Example of a Molecular Beacon melting curve. Temperature is decreasing left to right on the X axis, and fluorescence is plotted on the Y axis. The window between the two vertical blue lines represents a suitable annealing temperature range to discriminate two alleles with a 1-bp difference.

FIGURE 7: INTRODUCCION TO QUANTITATIVE PCR - STRATAGENE®

Scorpions probe chemistry functions in a manner somewhat similar to Molecular Beacons, but rather than having a separate probe, the hairpin structure is incorporated onto one of the primers. The fluorophore is attached to the 5′end of the primer and the 3′end is complementary to the target and serves as a site for extension initiation. A quencher is located between the primer and probe region of the oligo, so when the probe is in the hairpin configuration the reporter dye is located adjacent to the quencher. Following amplification and incorporation of the hairpin probe, the newly created strand is able to adopt a new structure. The loop sequence in the hairpin is complementary to the extension product of the probe/primer. During the subsequent round of denaturation and annealing, the loop sequence will anneal to the newly formed complement within the same strand of DNA. In this conformation, the fluorophore is separated from the quencher so fluorescence is produced. The primer also contains a "PCR blocker" in the hairpin which prevents the stem-loop structure from being copied during PCR by extension from the other primer.

Since the annealing of the loop sequence with the downstream PCR product is an intramolecular interaction, it is kinetically more favourable than probe systems which require two separate molecules to interact (the probe and template). For this reason, Scorpios typically result in higher fluorescence signal compared to TaqMan and Molecular Beacons. As with Molecular Beacons, Scorpios also do not rely on the 5′-3′exonuclease activity of DNA polymerase, so the reaction can be performed using a typical three-step thermal profile with the optimal extension temperature for the polymerase (72°C).

One disadvantage of the Scorpios chemistry is that the design and optimization of the probe structure is often much more challenging than with either Molecular Beacons or TaqMan probes, and, as a result, Scorpios are not generally suggested for those who are new to QPCR.

Primer and Probe Design

Primer and probe design is viewed as the most challenging step of setting up a new QPCR experiment. However, the availability of numerous primer and probe design software programs coupled with a set of easy to follow design rules makes the process relatively simple and reliable.

The first step in primer and probe design is to acquire the sequence of your gene of interest. Numerous publicly available sequences can be found in open access databases such as NCBI (www.ncbi.nlm.nih.gov). The Ensembl genome database (www.ensembl.org) provides transcript structures allowing identification of exon-intron borders, enabling the design of exon border spanning primers or probes when working with cDNA. For the design of primers and/or probes for Affymetrix microarray validation, the NetAff™. Analysis Center (www.affymetrix.com/analysis) is a valuable tool to identify GeneChip® array target regions on a given array.

After the sequence is obtained, a primer and probe design software program should be used in order to simplify and maximize success for the design process. Designer software packages are available both as freeware on the internet and through many oligonucleotide vendors. A representative list of primer design resources can be found in the Useful Websites section of this guide, or accessed from the MxPro software by selecting *QPCR Internet Links* from the Tools menu.

When using a software program to design primers and probes, it is important to set the concentration of monovalent ions (Na^+/K^+) and divalent ions ($Mg2^+$) to those that are used in your reaction for accurate melting temperature prediction. (The buffer conditions will generally be in the range of 50-100 mM monovalent cation and1.5-5.5 mM $Mg2^+$.)

The region of the template sequence to be used for detection must be considered carefully. The region of interest should be compared to the entire genome to ensure that the target sequence is unique [e.g., by performing an NCBI BLAST database search (www.ncbi.nlm.nih.gov/BLAST), and potential secondary structures should be identified and avoided [e.g., using the mfold program (www.bioinfo.rpi.edu/~zukerm/rna/).

For detection of coding sequence specific to RNA targets, it is advisable to design the probe to span exon-exon boundaries, thus preventing the detection of sequences from residual genomic DNA in the RNA sample. In circumstances where this is not an option, the RNA sample should be treated with DNase prior to the reverse transcription (RT) reaction. This is an efficient approach and results in minimal loss of sample when carried out on a column-based purification system.

In QRT -PCR, consider the method of cDNA synthesis when designing primers if oligo dT priming is used. It is generally safe to assume that the RT reaction has transcribed between 500-1000 bases from the polyA site with quantitative linearity, so it is best to design the assay to target a sequence for amplification towards the 3' end of the gene. The presence of SNPs and splice variants within a sequence should also be considered, as these must either be avoided or targeted as required according to the goal of the experiment.

For optimal performance, the region spanned by the primers (measured from the 5' end of each primer) should be between 70-150 bp in length for probe-based chemistries, and between 100-300 bp in length if SYBR Green I will be used. In order to maximize the efficiency of the PCR amplification, it is generally best to keep the target length relatively short. However, with SYBR Green I it is advantageous to use a slightly longer target so more of the dye molecules can bind to the amplified product and produce higher fluorescence signal. When designing for SYBR Green I with the intention of moving to a probe- based chemistry later, keep in mind to use the lower range (i.e., 100-200 bp) for primer design.

General rules for primers used in all chemistries are that each primer should be between 15-30 bp in length and the theoretical Tm of the two primers should be within 2°C of each other. It is best to try to avoid G/C clamps at the 3' ends of the primers to prevent these oligos from folding on themselves or annealing non-specifically. The five bases at the 5' terminal end generally should contain no more than two guanines and cytosines, although it is acceptable to have three in the final 5 bases if no two are adjacent. Since thymidine tends to mis-prime more readily than the other bases, a 3' terminal T should be avoided if possible. The 5' end of the primers should not contain an inverted repeat sequence that would allow it to fold on itself

In general, the Gibbs free enthalpy (□G) of primer dimmer and cross-primer dimer formation should be greater than -4 kcal/mol to ensure that primers do not form stable dimers. For multiplex reactions, it may be necessary to loosen the free enthalpy specification in order to allow for the design of the oligos required to work together in the same reaction. It is best to restrict the □G between each oligo pair to greater than -6 kcal/mol in a triplex reaction, and greater than -8 kcal/mol in a quadriplex reaction.

Probes should not contain runs of the same base (avoid more than three of the same base), and optimally should contain more "C" than "G" nucleotides. Guanine is an effective fluorescence quencher and should not be adjacent to the reporter dye.

Historically, TaqMan probes were situated 3-12 bp downstream of the primer on the same strand, but recent evidence suggests that the distance from the upstream primer to the probe is less important than previously thought. TaqMan probes are generally between 20-30 bp in length. Ideally they should have balanced GC content, although probes with varying content (20-80% GC) can still be effective. The Tm requirements of the probe will most often dictate the specific %GC.

TaqMan assays are conventionally performed as a two-step PCR reaction consisting of a product melt at 95°C, followed by primer annealing and Taq DNA polymerase extension at 60°C (Figure 8). For these assays the probe is designed with a Tm 8-10°C higher than the primer Tm's. Using the higher Tm for the probe ensures hybridization to the target before extension can occur from the primer, so there will always be a corresponding increase in fluorescence signal for every amplified copy that is produced.

Since TaqMan chemistry requires using the same thermal profile for each reaction, primers should always be designed with a Tm of approximately 60°C, and the hydrolysis probe with a Tm around 70°C. Optimization of the assay is

accomplished by adjusting primer concentration rather than optimizing according to annealing temperatures (this is detailed under the section entitled Primer Optimization Guidelines).

Molecular Beacon probes should be designed to anneal at 7-10°C higher than the primers, to allow hybridization before primer extension.

FIGURE 8: INTRODUCCION TO QUANTITATIVE PCR - STRATAGENE®

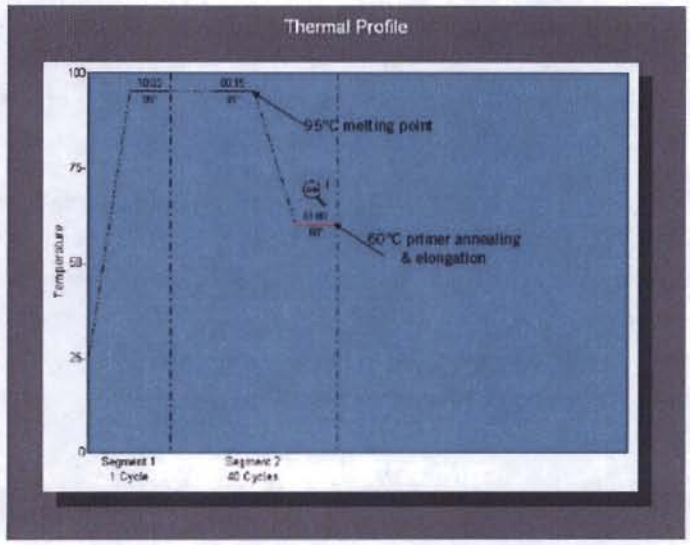


Figure 8. Typical two-step PCR thermal profile used for TaqMane QPCR reactions

For. Molecular Beacon, the stem sequence should .be designed to be 5-7 bp in length and should have a similar Tm to the melting temperature of the probe region in the .loop. As a general rule, stem sequences that are 5 bp long will have a Tm of 55-60°C, stems that are 6 bp long will have a Tm of 60-65°C, and stems that are 7 bp long will have a Tm of 65-70°C. Before having the Molecular Beacon probe synthesized, it is useful to use an oligo folding program [e.g., the mfold program (www.bioinfo.rpi.edu/-zukerm/rna/) to verify that the Pi sequence will form the desired stem-loop structure, using your specific salt conditions and annealing temperature. .Unlike TaqMan probes, Molecular Beacons are usually designed so that the probe is annealed closer to the midpoint between the two primers, rather than adjacent to the upstream primer. This will ensure that any low-activity extension by the polymerase at the annealing temperature will not displace the probe before the fluorescence reading is taken.

A Scorpions probe sequence should be approximately 17-30 bp in length. It is best to place the probe no more than 11 bp upstream of the complementary target sequence. The farther downstream this complementary sequence is, the lower the probe efficiency will be. The stem sequence should be about 6-7 bp in length, and contain sufficient pyrimidines so that the Tm of the stem loop structure is 5-10°C higher than the Tm of the primer sequence to the target, and the □G value for the stem loop confirmation is negative. The more negative the □G value, the more likely the folding will occur. Similar to Molecular Beacons probes, proper folding of the Scorpions probe should be verified using the mfold program listed above.

When designing probes, the combination and positioning of reporter dyes and quenchers is important. Make sure that the chosen quencher will efficiently suppress the fluorescence of your chosen reporter dye to ensure low background. Information on the recommended quenchers for each fluorophore is generally available from the companies that

synthesize these probes, and general guidelines on the choice of dark quenchers are given in .Dye and Quencher Choice section of this guide.

When designing primers and probes for multiplex reactions, adhere to the following additional rules: (1) all amplicons should be of similar length (±5 bp) as well as similar GC content (±3%) and (2) the primer set Tm's, as well as the probe Tm's, used in a multiplex assay should be within 1°C of each other.

For all QPCR reactions, it is a good idea to verify that all of the oligos (primers and probe) that will be used together in .the same reaction will not form dimers, particularly at the 3' ends. The 3' complementarity can be checked by scanning the sequences manually. If you are using primer design software, the program itself may run a check to make sure the sequence choices it picks are not complementary to each other.

Probe and Primer Synthesis

Primers used for QPCR can be synthesized with any oligo synthesizer or purchased from a commercial oligo house. Probes will also require the addition of dye and quencher molecules. Additionally, Molecular Beacon and TaqMan probes will need to be blocked at the 3' end to prevent them from acting as primers and producing extension products. An important consideration to take into account in the preparation of the primers and probe is how they are purified. It is best to consult the primer/probe manufacturer for guidance on the type and level of purification required for the oligo and application.

Once the oligonucleotides are received, it is best to aliquot them into smaller volumes before storing them at -20°C. Multiple freeze-thaw cycles can damage oligos, and probes with fluorescent tags are especially susceptible to this sort of degradation. Primers should be aliquoted into volumes that will not require that they be thawed more than 20 times, and probes should be stored in volumes that will result in no more than five freeze-thaw cycles.

After the probe has been ordered, an easy test that can be performed to ensure that the probe is quenching properly is to read the fluorescence from an aliquot of a probe, then perform a nuclease digestion of that aliquot and take a second fluorescence reading. Digesting the probe will free the fluorophore from quenching and you should see an increase of >5000 counts in the raw fluorescence signal. This digestion can be performed using 100 nM of probe in 25µl 1x buffer with 10U DNase or S1 Nuclease, incubated at room temperature for 30 minutes.

Dye and Quencher Choice

When designing a fluorescent probe, it is necessary to ensure that the fluorophore and quencher pair is compatible, given the type of detection chemistry. In addition, when designing multiplexed reactions the spectral overlap between the fluorophores and quenchers for the different targets should be minimized to avoid possible crosstalk issues (Figure 9).

For TaqMan probes, the most historically common dye/quencher combination is a FAM fluorophore with a TAMRA quencher. This combination will certainly work well, but in recent years dark quenchers have become more popular. Dark quenchers emit the energy they absorb from the fluorophore as heat rather than light of a different wavelength. They tend to give results with lower background, and are especially useful in a multiplex reaction where it is important to avoid emitted light from the quencher giving cross-talk signal with one of the reporter dyes. The most commonly used dark quenchers and the range of emission wavelengths at which they are efficient are:

Black Hole Quenchers®. (Biosearch Technologies):
BHQ-1 480-580 nm
BHQ-2 550-650 nm
BHQ-3 620-730nm

Iowa BlackTM Quenchers
(Integrated DNA Technologies):
Iowa Black-FQ 420-620 nm
Iowa Black-RQ 500-700 nm

A listing of TaqMan fluorophore and quencher combinations can be found at most oligo manufacturers' websites. Molecular Beacons have historically used DABCYL quenchers, which work with a wide range of fluorophores. However, Molecular Beacons can also be used with Black Hole Quenchers (BHQs).

In an Mx system, the choice of dyes will be limited by the filters you chose to have installed in the instrument. When multiplexing, you should choose dyes that are as spectrally distinct from each other as possible. In general, for duplex reactions the most popular combination is FAM and HEX (JOE™NIC™) with ROX™ as an optional reference dye. For triplex, FAM, HEX (JOE/VIC), and Cy™5 with ROX as an optional reference dye are suggested. For quadriplex, we suggest FAM, HEX (JOE/VIC), Texas Red®, and Cy5 dyes. Any fluorophores that have little to no spectral overlap are best suited for this type of application.

The Mx3005P® system contains five filters to accommodate multiplexing four targets with the reference dye, performing a five-target qualitative assay, or greater dye flexibility between reactions. The most common combination of four filters is FAM, HEX, ROX, and Cy5 (and all equivalent dyes in these filter sets). The fifth filter set will most often be Cy3, as oligo manufacturers offer several dye choices which work well with this filter. However, due the small spectral separation between HEX and Cy3 there can be some signal overlap with this pair. If running all five dyes simultaneously, the Alexa Fluor®. 350 (Alexa 350) filter set is commonly used instead of Cy3. The Alexa 350 filter set is in the far blue range of the spectrum and thus spectrally distant from all other filter sets. The two most common dyes used with this filter are Alexa Fluor 350 and AMC (Coumarin Blue). The AMC dye offers the advantage of being brighter and thus generating higher fluorescence signals.

As mentioned above, dark quenchers can be especially useful when multiplexing. TAMRA can be an effective quencher, but the emission spectrum for TAMRA does have some overlap with other dyes such as ROX, HEX, and Cy3. With Dark Quenchers, background from the quenchers will not be an issue.

FIGURE 9: INTRODUCCION TO QUANTITATIVE PCR - STRATAGENE®

Filter Set	Ex Wavelength	Em Wavelength
Alexa 350	350	440
FAM/SYBR Green	492	516
TET	517	538
HEX/JOE/MC	535	555
CY3	545	568
TAMRA	556	580
ROX/Texas Red	585	610
CY5	635	665
FR 640	492	635
FR ROX	492	610
FR CY5	492	665

Figure 9. Parameters of the Mx3000P® and Mx3005P® system filter sets. FR 640, FR ROX™ and FR Cy5.

Reference Dye Considerations

A reference dye is a fixed concentration of inert fluorescent dye (usually ROX) that is added at the same concentration in all the samples, usually by including it in the master mix or by adding it to the buffer stock solution before it is aliquotted into the individual reaction tubes. Reference dye normalization is performed by dividing the raw fluorescence signal for each reporter dye at any given cycle by the raw fluorescence signal of the reference dye at the same cycle and then drawing the amplification plot based on these ratio values.

Historically, reference dye has been used to correct for sample to sample signal variation that is not due to the chemistry itself (e.g., aliquotting errors or deficiencies in the signal uniformity due to the instrument optical system). In the Mx system, the scanning read-head ensures that all wells receive the same level of excitation light and have the exact same light path to the detector so there is no need to correct for positional differences. Also, baseline correction algorithms generally correct for most variation due to aliquotting errors without the need for reference dye normalization, which makes the use of a reference dye optional in the Mx system.

Running a reference dye can still have some value, because in some cases it can result in somewhat cleaner looking data. Also, even if normalization is not performed it can be very useful in the troubleshooting process if you see any unexpected results in the amplification plots of your reporter dyes. If signal is particularly low or high, or if there is an odd shift in the fluorescence level, you can check to see if similar effects are seen in the reference dye profile, which should normally run flat. Whether effects are seen only with your reporter dye or with both the reporter and reference dyes can often allow you to discriminate probe problems from potential instrument problems.

If ROX is used as a reference dye, from an instrument standpoint there is a difference in the concentration of reference dye that should be used in the Mx instrument vs. some other systems. The white light excitation in the Mx system and the system's dye-specific filters will excite and measure the fluorescence for ROX very efficiently. Stratagene designed the ROX filer set to be very sensitive so this dye channel can be used for actually detecting fluorophores labeled with ROX or Texas Red. In systems that do not allow excitation at -584 nm (including laser-based systems), ROX is excited very inefficiently, so a higher concentration of the reference dye is used to compensate for the low ROX signal. If a kit that is designed for one of these systems is used in the Mx system, the high concentration of ROX will create oversaturated signal on the ROX channel and result in the normalized data containing more noise than the non-normalized data. In the Mx system, ROX should be used at a final concentration of approximately 30 nM of free dye. If a master mix containing a final ROX concentration closer to 300 nM is used, it is recommended that the non-normalized baseline-corrected amplification plots (dR) be used for analysis rather than the normalized baseline-corrected plots (dRn).

Some master mixes contain a short oligonucleotide labeled with FAM and ROX that causes emission from ROX by energy transfer or FRET. The presence of these oligonucleotides is compatible with fluorescence detection in the Mx system and should not cause any difficulty in normalization.

Controls for Quantitative PCR Experiments

Ultimately, the objective of using real-time quantitative PCR experiments is to determine the absolute quantity of the target sequence present in the sample or to monitor the fold changes of genes in response to experimental conditions. For accurate data analysis and meaningful statistics using either of these approaches, the appropriate positive and negative controls must be included with each real-time assay.

The specific controls that are needed will vary according to the experiment type, but there are certain controls that should be included in every run, such as No Template Controls (NTC). The two primary types of controls are .positive controls and negative controls. Positive control samples should always show amplification, and if they fail to show expected amplification, it indicates some sort of problem with the assay or reagents. Negative controls should not show any amplification, and if an increase in fluorescence is seen it would indicate such things as contamination, non-specific PCR product formation, or non-specific probe degradation. For qualitative experiments in particular, if your experimental parameters require that you avoid false positives you should always run negative controls, and if you need to ensure that you avoid false negatives you should always run positive controls.

Positive Controls

Positive controls will assist in identifying false negatives that might occur due to sample template quality, PCR inhibitors, etc., typically associated with heterogeneous samples (e.g., applications such as pathogen detection, GMO testing, or mutation detection). In these experiments, inclusion of positive controls in all the amplification reactions validates the absence of detection of the target of interest as a true negative sample. In reactions where no positive control is run, if all your unknown samples come up negative, it is impossible to tell if these are true negatives or if some problem in your reagents caused amplification to fail.

Positive controls can be either completely different samples that are not related to your experimental samples (referred to here as exogenous positive controls), or they can be separate targets amplified from the same sample of nucleic acid (referred to here as endogenous positive controls). Exactly what type of positive control is used will control for different types or reaction failure.

An exogenous positive control is a source of the template that is known to contain the target of interest but which is independent of your experimental samples. Examples would be plasmid containing the gene of interest, in vitro RNA transcripts, previous PCR products, Human or Mouse QPCR Reference Total RNA, or DNA or RNA isolated from organisms known to contain or express the target of interest. Controls of this sort are very useful for just verifying that the amplification reaction is working and the fluorescence signal is being generated and detected normally. They will not reveal if there are PCR inhibitors that are carried along with the nucleic acid in any of your unknown samples or if there is degradation of the nucleic acid in some samples.

The use of an endogenous positive control involves amplifying a second target from the same sample as the gene of interest. This can either be run in multiplex or in a separate tube containing an aliquot of the same template sample. If a normalizer gene is being used in the experiment (detailed below in the section on Normalization) this will serve as a positive control of this sort. Any gene that is known to be present in the experimental sample (or any RNA known to be expressed, in the case of QRT -PCR) will work as a normalizer-type of positive control. Endogenous positive controls will alert you to general problems with the buffer or polymerase just as a positive control sample will, but

since they must use a different primer/probe set than the gene of interest they cannot be used to detect problems resulting from those reaction components.

Controls of the sort described so far will only provide you with a yes/no answer on whether amplification is taking place, and will not detect an RT or PCR inhibitor present in the sample preparation that is causing a low level of inhibition, and delaying Ct values without preventing amplification. Another type of positive control is an exogenous target that is spiked into the template sample, such as Stratagene's Alien® QRT -PCR Inhibitor Alert target RNA. The Alien RNA Transcript is spiked into the purified RNA sample prior to reverse transcription and is amplified using Alien transcript-specific primers. QRT -PCR of the Alien control target is also run alone, in a separate tube, alongside the samples of interest. Measuring amplification of the control target side by side in both samples can be used to detect PCR inhibitors that influence the reaction Cts without preventing amplification outright. Figure 16 shows detection of QRT-PCR inhibition by guanidine using the Alien QRT-PCR inhibitor alert kit. This sort of positive control does not control for RNA isolation variances, which is controlled for using a normalizer gene.

Positive controls can be used to provide consistent positive reference data points in a given experiment. A standard curve can be generated by simply performing a dilution series on the positive control material. If a standard curve of the gene of interest is performed in a given run and the standard template concentrations span the range of expected unknown sample concentrations, the MxPro software can use this to perform absolute quantification of unknown samples by plotting the Ct values of the unknowns on the standard curve. Some examples of appropriate positive control templates include plasmids containing the target sequence, purified PCR products, synthetic oligonucleotides, and Stratagene's QPCR Human or Mouse Reference Total RNA. As described later, standard curves can also be used to calculate the exact efficiency of amplification in your QPCR reaction. A decreased amplification efficiency or loss of linearity in the standard curve may indicate that a problem has developed with your reagents or that there is a contaminating inhibitor. In this case the standard curve will provide a useful indicator of whether or not the experimental data is valid, and to what degree it can be compared to samples performed in separate runs.

Negative Controls

Negative controls are often overlooked in experimental design, but they are one of the most important components of a QPCR-assay. A negative control will typically be missing one of the components essential for the reaction to proceed, and thus it is expected to yield no shift in fluorescence. Depending on the type of negative control that is run, you can test for problems that might occur in the reaction at multiple different steps. In addition to assay-specific negative controls, which use template samples from sources that are expected not to contain the sequence of interest, other common types of negative controls are no template controls (NTC), no reverse transcriptase controls (No RT), no amplification controls (NAC), and no probe controls (NPC).

NTCs provide a mechanism to control for external contamination or other factors that can result in a non-specific increase in the fluorescence signal. Ideally, signal amplification should not be observed in the NTC sample wells. If the NTCs do cross the threshold, their Cts should be at least five cycles, and preferably more than ten cycles, from the Cts of your least concentrated samples. If the Cts of the NTCs are less than five cycles delayed compared to samples containing template, the Cts of those samples should not be considered accurate since whatever is causing the fluorescence shift in the NTC wells could also be affecting the fluorescence in the unknown wells.

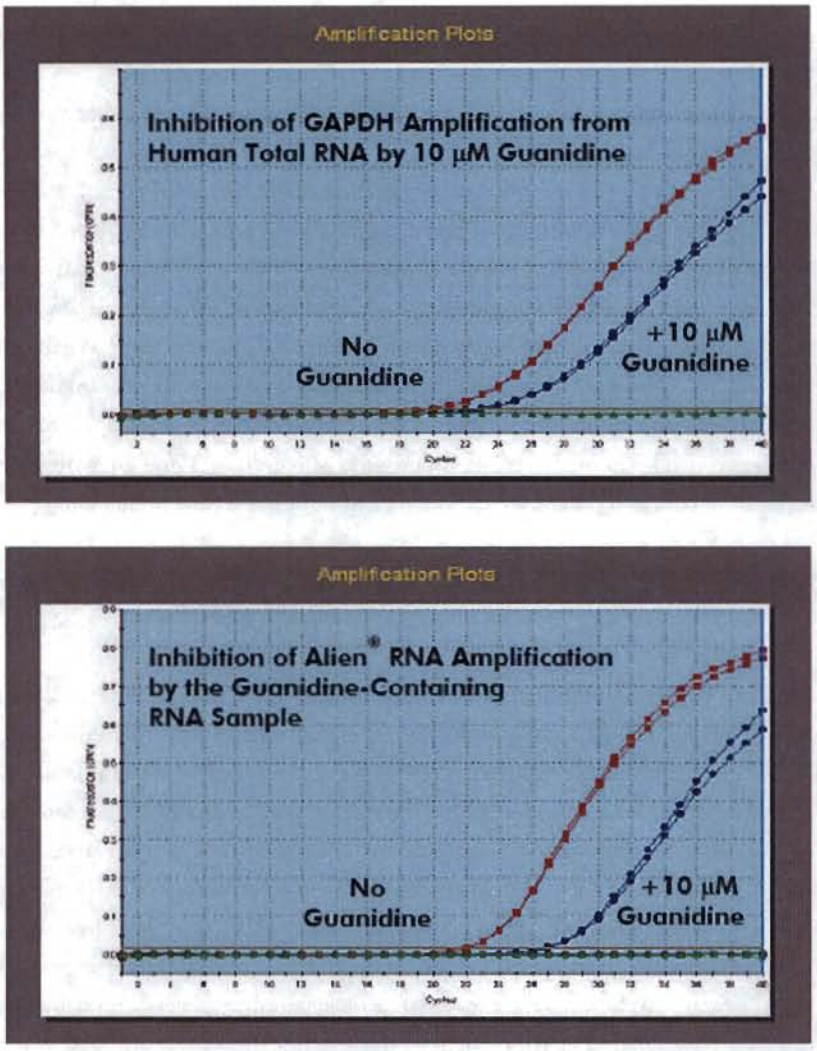


Figure 10. Detection of inhibition by 10 µM guanidine using the Alien® QRT –PCR Inhibitor Alert. The upper panel shows amplification of the GAPDH target from human total RNA (50 ng Stratagene® QPCR Reference Total RNA. Human) in the absence and presence of 10 µM guanidine (final concentration in the QRT -PCR reaction). The lower panel shows amplification of the Alien-RNA target from samples containing a mixture of 10^5 copies of Alien® RNA transcript and 50 ng human total RNA, in the absence and presence of 10 µM guanidine (final concentration in the QRT- PCR reaction). In the presence of 10 µM guanidine, a delay of 3 Ct values was observed for the GAPDH target, and a delay of 5 Ct values was observed for the Alien® RNA target. Experiments were performed using the Brilliant® SYBR® Green I-Step QRT-PCR Master Mix.

No RT controls are samples that are run exactly as the other QRT -PCR reactions, except that the reverse transcriptase enzyme is omitted. No RT controls should show no amplification in the subsequent PCR step since DNA polymerase cannot amplify an RNA template. Amplification occurring in the No RT control wells indicates that there is contaminating DNA template in the reaction. If amplification is observed in the No RT controls and not in the NTC reactions, contaminating genomic DNA is most likely present in the RNA sample.

Another common negative control is no amplification controls (NAC) which includes all the reaction components except for the DNA polymerase. This is useful if you suspect that an increase in fluorescence in your reaction is due to something other than actual amplification (e.g., your probe is degrading).

No probe controls (NPCs) are useful to test for background fluorescence signal, possibly due to contamination, but are .rarely used. When using a probe-based chemistry like Taqman or Molecular Beacons, the fluorescence signal is generated by the fluorescent dye molecule on the probe. In .reactions lacking probe, you should see the true background fluorescence level. .

When performing qualitative PCR (i.e., generating a positive or negative determination of whether or not a given t sequence is present), it is necessary to include at least three NTC wells, or three dye-specific negative control wells, in order to determine statistically whether or not real amplification has occurred. The MxPro software bases a positive or negative call result on a p-value test, and a minimum of three negative data points are required to produce the population that the unknown wells will be compared to in order to determine if they are significantly amplified. The p-value is the probability that the mean of one set of sample data is different from the mean of another set of sample data. The first set of sample data is always the negative control wells in the analysis selection. When replicates are being treated individually, the second set of sample data consists of a single well (usually an Unknown well). When replicates are being treated .collectively, the second set of sample data consists of all of .the replicates. If the p-value exceeds the user-specified confidence level, the well/dye is called as positive and t signified with a plus sign (+). Otherwise, the well is called as negative (i.e., no difference detected) and signified with a minus sign (-).

Passive Reference Dye

Although it is not an amplification control, it is common practice when performing QPCR to include a reference dye in the reaction mixture. The reference dye is not linked to any amplification effect. Therefore, the fluorescence from this dye should be constant throughout the amplification reaction. Provided concentration and volume are equal in every well of the reaction, theoretically the fluorescence -intensity for the reference dye should be the same in every sample. The fluorescence signal for the fluorophores in the reaction can be normalized to the reference dye by dividing the raw fluorescence intensity at each cycle for the dye of interest by the fluorescence intensity from the reference dye at the same cycle in the same tube. This will act to correct or "normalize" any signal level differences (e.g., those caused by differences in plasticware transparency .and reflectivity, or volume differences due to aliquotting errors). This correction is not required, but if a reference dye is designated, it is performed automatically by the .MxPro software. Corrected data are designated as Rn or dRn in the amplification plots and text report. The most .commonly used reference dye is ROX. Pure ROX dye should be used at a final concentration of 30 nM in the Mx system.

Assay Optimization

To ensure efficient and accurate quantification of the target template, QPCR assays should be optimized and validated for quantitative range and specificity. This process requires the use of .an abundant and quantifiable control template. The most common source of control templates is target that has been cloned into a plasmid (the recombinant plasmid must be linearized and purified), a purified PCR fragment, genomic DNA, a synthetic oligonucleotide, or a stock of cDNA from cell culture. When selecting a. control template for assay optimization, the goal is to select a material that may be obtained in high abundance, which is most like the unknown samples you intend to analyze, and that contains the sequence of interest. If a linearized plasmid or oligonucleotide control template is used as the template, frequently this will require spiking in carrier material such as genomic DNA, yeast tRNA, or glycogen, to replicate sample complexity and avoid sample loss.

Assays are most easily optimized by first evaluating the primer concentrations (forward and reverse) for a given template, across a range of concentrations. This requires the use of a standard curve, or a linear dilution series of starting material, to determine assay quality metrics. These metrics include QPCR efficiency, precision, sensitivity, and specificity, and can be assessed using a standard curve and SYBR Green I detection chemistry.

Following primer optimization, it may be necessary to further optimize the probe concentration (if the assay uses probe-based detection chemistry) and the Mg^{2+} concentration. Completing the initial primer optimization step using SYBR Green I detection chemistry is generally sufficient to ensure an efficient reaction for other chemistries while conserving the probe to reduce cost.

Primer Optimization Guidelines

Depending on the QPCR chemistry being utilized for the assay, different ranges of primer concentrations can be tested. For SYBR Green I, relatively low primer concentrations are used to avoid primer-dimer formation. For most SYBR Green I applications, primer concentrations ranging from 50-300 nM are appropriate.

For sequence specific probe chemistries like TaqMan and Molecular Beacons, a wider range of primer concentrations needs to be considered. Typically, primer concentrations ranging from 50-900 nM should be tested.

However, not all assays require the testing of this entire range of primer concentrations. Starting out with a new primer set for a gene expression experiment, for example, one might try 300 nM of each forward and reverse primer, with a typical serial dilution. This would consist of five points of a five-fold serial dilution, starting with 100 ng of total RNA per reaction (or the equivalent cDNA amount). Inclusion of a negative control of just the primer (at each concentration) in the absence of template (NTC) will yield specificity information. A successful assay will have good linear Cts versus input amount of template, as indicated by the metrics described below, as well as a melt curve that predicts a single product in template positive samples, and a negative result for the NTC.

To demonstrate more complex primer optimization strategies, this guide will illustrate the optimization matrix for primer concentrations from 50-600 nM. These reactions should be run in duplicate with the appropriate negative controls for each concentration. Ideally, a middle concentration of template (5-10 ng RNA) should be used to assess each of these primer concentration pairs with SYBR Green I (Figure 14).

The ideal primer pair will yield the lowest average Ct, as well as a melt curve that shows a single product for the positive template sample and a negative result for the NTC.

Primer Optimization with SYBR® Green I

SYBR Green I is inexpensive and easy to use, making it ideal for use in primer optimization. Since SYBR Green I dye is a DNA binding dye, it will generated signal from both specific and non-specific products. The generation of all products can be easily visualized on a melt curve following the amplification reaction. Therefore; SYBR Green I dye can be used to determine both primer performance and primer specificity at different concentrations. As a result, the entire primer optimization process can be completed independently before ordering the sequence-specific probe. This is desirable because if the primers are not working it may be necessary to redesign them. Since this may also involve redesigning the probe, it is worthwhile to run this test prior to ordering the probe.

Primer Optimization Data Analysis

Using the MxPro software, you can analyze dR or dRn. Analysis of dRn is only applied if a passive reference dye (e.g., ROX) is used in the experiment. It is recommended that a passive reference dye be used, as it tends to improve data quality. If no reference dye is used, then analyze dR. In the following examples dRn is used. Once the run is completed, examine the Ct and dRn Last values for each primer combination. Select the primer combination that results in the lowest Ct value and the highest dRn Last value. When optimizing primers using SYBR Green I, it is also crucial to analyze the melt curve data for each primer concentration pair to ensure a single homogenous product is being generated. If several primer combinations give very similar results, pick the primer combination with the lowest overfill concentration.

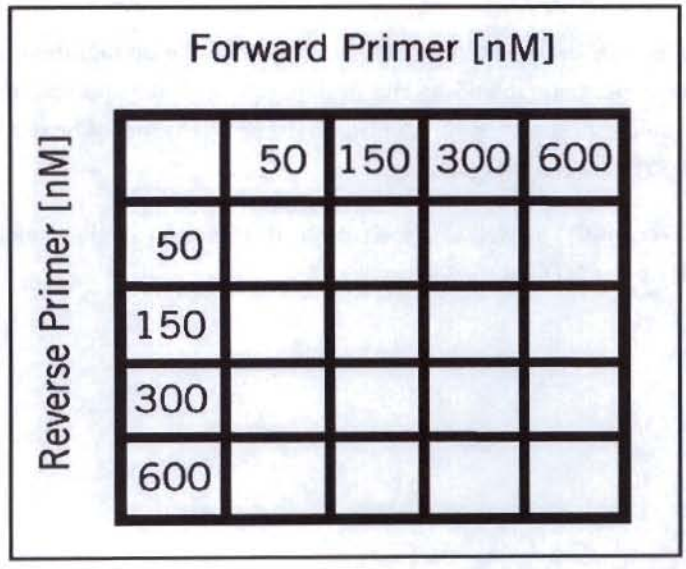


FIGURE 11: INTRODUCCION TO QUANTITATIVE PCR - STRATAGENE®

Figure 11. Primer optimization matrix. For each primer pair, the Ct should be determined, and the dissociation curve should be analyzed to verify a single product for the template-containing samples and no product for the no-template controls (NTCs).

Dissociation (Melting Curve) Analysis

During a Melting Curve Analysis, all products generated during the PCR amplification reaction are melted at 95°C, then annealed at 55°C and subjected to gradual increases in temperature. During the incremental temperature increases, fluorescence data are collected until the reaction reaches 95°C. The result is a plot of raw fluorescence data units, R, versus temperature (Figure 11.1). This view of the data may appear difficult to interpret at first, but the rapid linear decrease in fluorescence to background is where the major PCR product melts to its single stranded form.

Figure 12 shows the melt data as the negative first derivative of raw fluorescence, R'(T), vs. temperature. This is the easier view to identify the Tm of each product, indicated by the peak in R'(T). In analyzing the various combinations of primer in Figure 12, you can see one major peak at 78°C for the majority of the samples, but a few samples have peaks shifted to 79°C. The distinct melting peaks may indicate multiple PCR products in this assay. A fully optimized assay should contain only a single melt product. Banding by agarose gel analysis of the PCR product will determine if the appearance of more than one Tm indicates more than one product, or rather an artifact of the SYBR dye binding. Alternative splice forms, insertions, or deletions could create alternatively melting PCR products, from specific priming sites. The Cts from such assays should be scrutinized, and no meaningful quantification should be based on these data.

Two steps are required to interpret results from a SYBR Green I melt curve analysis. The first step is to review the PCR products produced by the samples in the reaction. In the example shown in Figure 13, the presence of a single homogeneous melt peak for all sample reactions confirms specific amplification. The data from this reaction are reliable and meaningful for analysis and interpretation. The second step is to evaluate the NTC sample well for the presence of primer-dimer formation. Slight, high cycle amplification and a small wide peak at a lower temperature by melt is an indication of primer-only amplification. It is acceptable to observe a small amount of primer-dimer formation in the NTc wells, but if there is a corresponding peak in the sample amplification plots the Cts from these wells cannot be trusted as accurate.

Choosing the Correct Primer Concentration

Figure 14 shows an example of a 50 nM-600 nM primer matrix in the presence of a linear hydrolysis probe. Based on Ct only, the primer concentration combination of 150:300 nM (forward:reverse) gives optimal but comparable results to other concentration pairs. It is best to get the lowest Ct values possible, but it is often as important to reduce the overall primer concentration if you are planning to use this as part of a multiplex assay. When multiplexing, the lower the overall concentration, the less chance that the reactions will interfere with one another.

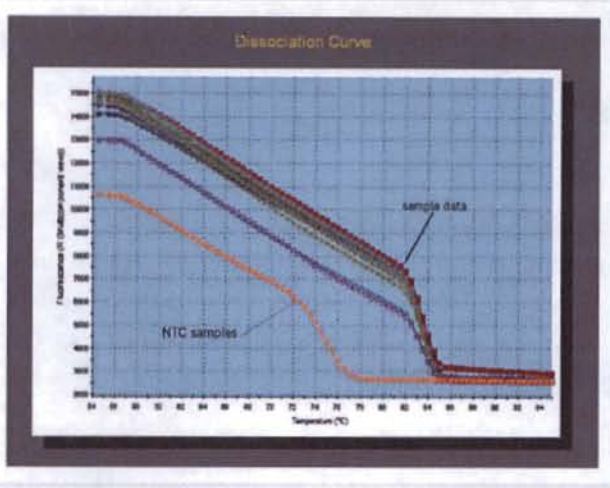
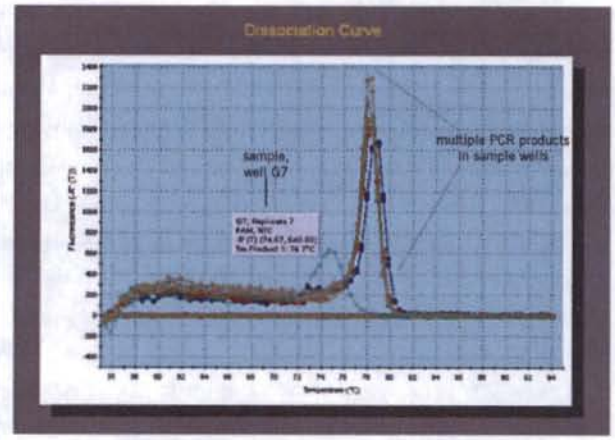


Figure 11.1. Raw fluorescence signal change plotted as a function of increasing temperature. The higher traces show a rapid melt between 82°C and 84°C. The NTC samples show a change in plot shape around 72°C.

Figure 12. The negative first derivative of raw fluorescence plotted against increasing temperature during the melt curve. Sample well G7 is highlighted to indicate a small amount of primer-dimer product melting in the NTC sample. The other plots indicate melts at 78°C and 79°C, indicating two PCR products in some of the sample wells.



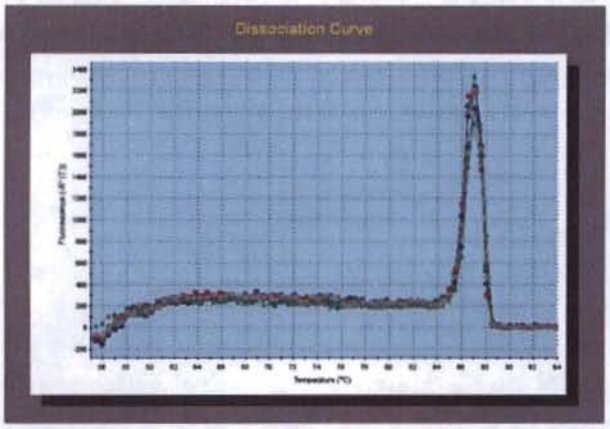


Figure 13. The first derivative of raw fluorescence plotted against an increase in temperature. The single melt peak at 86.5°C indicates a single PCR product is being amplified in these samples.

After analyzing the amplification plots, 150 nM forward primer concentration and 300 nM reverse primer concentration was chosen because this combination produced the lowest Ct and highest dRn. Additionally, when primers are individually optimized on the basis of concentration, there is a greater chance that they will function optimally in a multiplex format with other primers similarly optimized. Traditionally, primers could be optimized by changing annealing temperature in the assay thermal profile, however, this strategy is not appropriate for multiplex assays. Since a multiplex assay is run at one consistent thermal profile, it is unlikely that one optimal annealing temperature will be found where all primer concentrations function optimally. Thus, individual primer pair optimization on the basis of concentration using a constant thermal profile is favored.

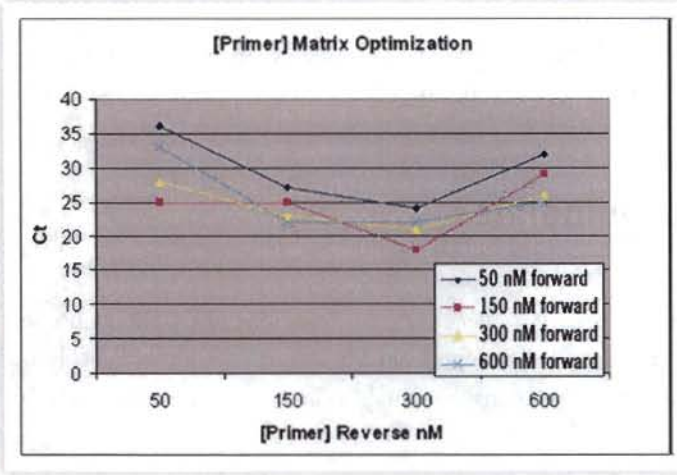


Figure 14. Analysis of Prime optimization matrix data. Cts for each of the primer concentration pairs given in Figure 11 are plotted.

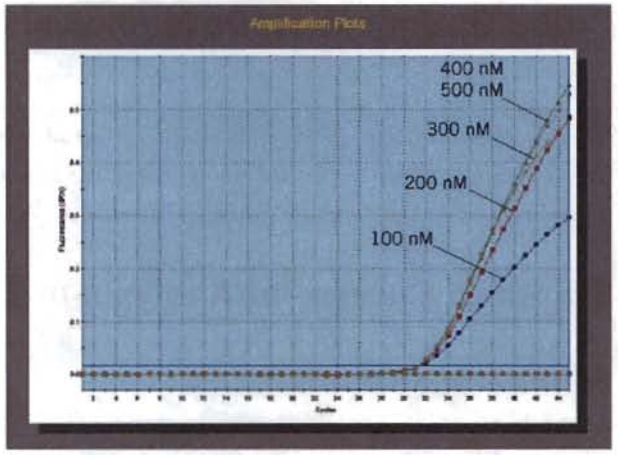


Figure 15. Probe optimization data plotted as cycle number vs. dRn fluorescence. All probe concentrations generate the same Ct value. However, the dRn Last values are decreased significantly with 100 nM probe, and decreased only slightly with 200 nM and 300 nM of probe. Based on these data, 200 nM of probe would be optimal.

Primer Optimization with Fluorescent Probes

If you prefer to optimize the assay with a fluorescent probe in each phase of the process, the first step is still to determine the optimal primer concentrations. A good starting concentration for linear hydrolysis probes is 200 nM although lower concentrations of 100 nM can be used if probe quantity is a concern.

The procedure for performing the primer optimization matrix experiment using probe-based detection is nearly identical to that listed for the SYBR Green I procedure (see Figure 14). The one major exception in this approach is the thermal profile to be used for the linear hydrolysis probe experiment. Linear hydrolysis probes (TaqMan probes) use a two-step thermal profile, and Scorpions or Molecular Beacon probes use a three-step thermal profile with pre-determined optimal annealing temperatures. Fluorescent probe thermal profiles do not employ a melt curve like that of a SYBR Green I assay.

Primer Optimization Data Analysis

Analysis of probe-based primer optimization is similar to SYBR Green I primer optimization analysis, but does not include the melt curve component. Optimal primer combinations are still determined by the lowest Ct value and highest dRn Last value.

Probe Concentration Optimization Guidelines

After the optimal primer concentrations have been determined, it is necessary to determine the optimal probe concentration for the assay. Fluorescent probe concentrations typically range from 50-300 nM for linear hydrolysis probes and Molecular Beacons, while other QPCR chemistries, like Scorpions, might require concentrations as high as 500 nM.

Probe Optimization Data Analysis

Analysis of fluorescent probe optimization is similar to primer optimization analysis. Using the optimal primer concentrations, select the probe combination that results in the lowest Ct value and the highest dRn Last value. If several probe combinations give very similar results, pick the lowest probe concentration (Figure 15).

Standard Curves for Analysis of QPCR

Assay Performance

After determining optimal primer and probe concentrations for the assay, we recommend testing the overall performance of the QPCR reaction in terms of efficiency, precision, linear range of quantitation, and sensitivity. Data generated from a serial dilution of a positive control template (standard curve) are an excellent means of determining the overall performance of a QPCR assay. The dilution series should encompass a large range of concentrations to ensure the reaction performs at equal efficiency for high and low concentrations of starting template, ideally encompassing the expected levels of target to be encountered with the experimental samples. To accomplish this objective, a three-fold to ten-fold dilution series over several orders of magnitude should be generated in triplicate. For example, for gene expression experiments, a typical serial dilution would consist of five points of a five-fold serial dilution, starting with 100 ng of total RNA per reaction (or the cDNA equivalent amount). If the assay is intended to quantitate genomic DNA or copy number, such as with viral quantitation assays, a starting concentration of purified plasmid or PCR product in the 10-25 ng range is adequate. Be aware that not all points of a standard curve will conform to high data quality metrics as described below. Often, the high and low concentration points may not be in range, and elimination of these aberrant concentrations from analysis may result in a high quality assay, across a slightly lower linear quantitative range.

PCR Reaction Efficiency

The slope of the line of best fit drawn to the standard curve is used to determine reaction efficiency. The standard curve plots the log of starting template vs. PCR cycle number, and is generated by the MxPro software. A linear fit with a slope between approximately -3.1 and -3.6, equivalent to calculated 90-110% reaction efficiency, is typically acceptable for most applications requiring accurate quantification. If the amplification reaction is not efficient at the point being used to extrapolate back to the amount of starting material (usually the Ct is used for this purpose), then the calculated quantities may not be accurate. Since the PCR reaction is based on exponential amplification, if the efficiency of PCR amplification is 100%, the amount of total template is expected to double with each cycle. This assumption allows the reliable calculation of quantity from Ct, and thus -100% QPCR efficiency needs to be assessed and verified prior to running valuable samples.

Precision

The standard curve should be run in triplicate (or at least duplicate) so that it is possible to determine the precision of pi petting, the reproducibility, and the overall sensitivity of an assay. Rsq is the fit of all data to the standard curve plot and can be influenced by accuracy of the dilution series, and overall assay sensitivity. If all the data lie perfectly on the line, the Rsq will be 1.00. As the data fall further from the line, the Rsq decreases. As the Rsq decreases it is more difficult to determine the exact location of the standard curve plot thus decreasing the accuracy of quantification. An Rsq value >0.985 is acceptable for most assays.

Sensitivity

The slope and Rsq values of the standard curve help determine the sensitivity of a given assay. If the slope of the standard curve is lower than -3.322 (100% Efficiency), the Rsq is below 0.9851, and the data points indicate an upward trend in the standard curve plot at the lower template concentrations, this may indicate the reaction is reaching the threshold of sensitivity, i.e., more cycles are required to amplify ever decreasing amounts of template. In this case, further assay optimization or even redesign of the primers and probe may be necessary to extend the linear range. Alternatively, the points outside the linear range can be culled from the standard curve. However, unknown samples in that concentration range may not be trusted to give quantitative interpolation from that part of the standard curve, or Cts from that range should not be used in further analysis.

Standard Curve Examples

Figures 16 and 17 illustrate a four-fold dilution series standard curve over three orders of magnitude. In this example the data generate a linear standard curve with a slope of -3.401 (96.8% Efficiency) which is well within the acceptable range, and an Rsq value of 1.0.

Further Optimization

If the assay is still not performing well after the probe and primer concentrations are optimized, you can try altering the Mg^{2+} concentration in the reagents by adding extra $MgCl_2$ from Stratagene's core reagents kits. Increasing the Mg^{2+} concentration tends to favor hybridization and therefore excessive Mg^{2+} can promote the formation of primer-dimers as well as template specific priming. While primer-dimers are not detected by sequence specific probe chemistries, they can cause the reactions to be inefficient and therefore less sensitive and detect fewer positive samples.

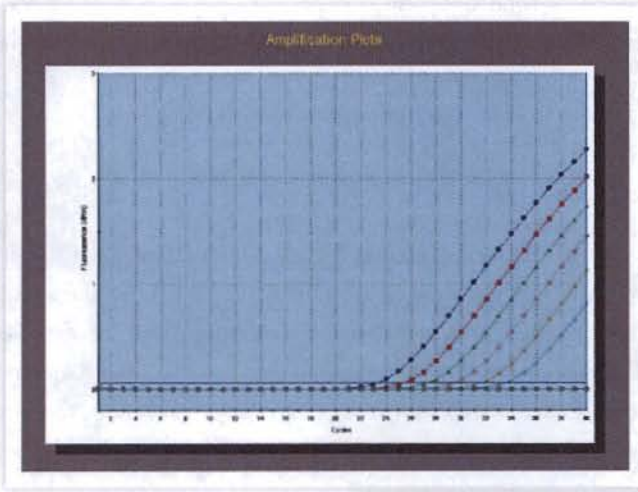


FIGURE 16: INTRODUCCION TO QUANTITATIVE PCR - STRATAGENE®

Figure 16. Amplification plots of standards in a four-fold dilution series over three orders of magnitude.

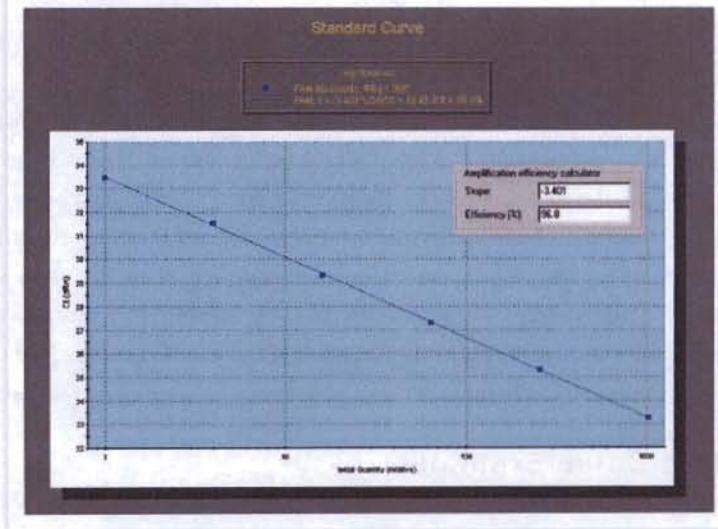


Figure 17. Standard curve generated with data from Figure 20, with slope and Rsq indicated.

FIGURE 17: INTRODUCCION TO QUANTITATIVE PCR - STRATAGENE®

In most experiments, it is sufficient to use a standard concentration of $MgCl_2$, depending on the type of QPCR chemistry employed in the assay. For linear hydrolysis probes (TaqMan), begin with a final concentration of up to 5.0 mM $MgCl_2$ Molecular Beacons use a lower concentration of 3.5 mM. Scorpions use a lower concentration of 1.5 mM to 2.5 mM $MgCl_2$. In SYBR Green I assays, primer-dimer formation and detection can contribute to the overall signal. Therefore, for SYBR Green I assays it is best to use 1.5 mM to 2.5 mM $MgCl_2$ to avoid excessive dimer formation.

When multiplexing, a low standard Mg^{2+} concentration can also be used to avoid cross reactivity of primers and probes.

If the reaction still does not work well after complete optimization is performed, it may be necessary to redesign the primers and/or the probe.

Multiplex Assay Considerations

If the experimental project requires many runs over time on the same set of genes (e.g., time course studies, metastasis progression research), it may be more cost effective and provide a higher level of statistical correlation to design a multiplex reaction to use for the duration of the study. The multiplex approach is particularly important when the template material is limited because this allows the maximum amount of data to be generated from each assay. It does require- more up front expenditure in the form of probes, and more time to design and optimize reactions that will all work together in the same tube, but the long term savings in reagent costs, plasticware, and time from the reduced number of experimental runs can be substantial.

The primers and probes for multiplex QPCR reactions are designed the same as they would be for singleplex reactions, with a few extra considerations. All of the primers and probes that will be used in the same reaction should be of similar length, Tm, and GC content. Also, special care should be given to ensure that none of the oligos will interact with one another. In a singleplex reaction, the ΔG value for any two of the oligos in the solution should be -2 or greater (more positive). This suggests a lower probability that the two primer oligos will energetically favor hybridization, over hybridization to the specific template. This may not always be possible for the large number of oligos in a multiplex assay, but minimally you should try to achieve ΔG values in the following ranges:

Singleplex: Greater than -2
Dup.tex: Greater than -4
Triplex: Greater than -6
Quadriplex: Greater than -8

When optimizing the relative primer concentrations, it is especially important with a multiplex assay to use the lowest primer and probe concentrations possible. The higher the oligo concentrations, the greater the chance the reactions will interfere with one another. Standard curves should be run during the assay optimization for all the reactions, in both singleplex and multiplex, to ensure that the reactions do work together. The efficiency in the singleplex reactions and the multiplex reactions should not differ by more than 5%, and the Ct values should not change by more than approximately 1 Ct.

If the multiplex assays do not appear to be working well together, it may be necessary to add additional reaction components to ensure that reagents are not limiting to the multiple reactions. In theses cases, the Taq DNA polymerase increase and dNTP concentrations can both be increased by between 50-100% and the buffer concentration can be increased from a 1x solution to a 1.5x solution. For this purpose, Stratagene offers a Brilliant Multiplex QPCR Master Mix that is optimized for the simultaneous amplification of multiple targets.

QPCR Experiment Data Analysis

Ensuring Your Ct Values are Accurate

After the data are collected, it is best to examine them carefully to ensure the run went well and the assigned Ct values are accurate before you start looking at the calculated absolute or relative quantities. Immediately after the run completes, it is best to remove the sample tubes from the instrument and examine them. Verify that the caps are properly in place and that no loss of liquid due to evaporation is noticeable. If evaporation has occurred any anomalous wells can be culled from the analysis.

When first analyzing your amplification plots, you should follow these steps:

1. Look at the raw fluorescence values.
2. Check the baseline settings.
3. Check the threshold.
4. Look at the dissociation curves (if SYBR Green I was used).

Dissociation Curves (Only for SYBR@ Green I)

As mentioned previously, when the detection chemistry is based on double-stranded DNA detection, such as SYBR Green I, you should run a melting curve at the end of your amplification reaction known as a dissociation curve. The purpose of the dissociation curve is to determine if anything other than the gene of interest was amplified in the QPCR reaction. Because SYBR Green I will bind any double- stranded product, any non-specific amplification in your unknown wells will artificially increase fluorescence and make it impossible to accurately quantitate your sample.

To view the SYBR Green I dissociation curve, select the Results tab, and under Area to Analyze go to 'Dissociation curve' in the software. The best way to analyze the dissociation curve results is to set the fluorescence to -Rn'(T), although if you have not run a normalizing dye you should set this to -R'(T). In this view, every peak in the curve indicates a specific product melting. Most QPCR products will melt somewhere in the range of 80-90°C, although this can vary given the size and sequence of your specific target. Ideally, you should see a single peak within this temperature range, and the melting temperature should be the same in all the reactions where you have amplified the same sample. If any secondary peaks or shoulders are seen on the peak of interest, it indicates that something other than your gene of interest is present among the reaction products. Since there is no accurate way to determine how much the amplified signal from each product is contributing to the Ct, if any secondary peaks are observed the Ct value from that well should not be considered accurate.

If secondary peaks are seen, other controls run in the reaction may give you an indication of what is causing this problem and how it can be prevented in the future. If these same secondary peaks are present in your NTC wells, it may indicate primer dimer formation or the presence of contamination by a sequence that was also amplified during the reaction. Since primer dimers will typically have a lower melting temperature, the temperature at which the peak occurs can generally be used to discriminate peaks caused by primer dimers from peaks due to amplicon contamination. In the case of primer dimers, re-optimizing the reaction conditions may be necessary. On occasion, it

may be necessary to re-design the primers. If the secondary peaks are not seen in the NTC wells, it could indicate non-specific primer binding or the presence of differentially spliced products. Performing a BLAST search following primer design can help decrease the incidence of this type of problem.

Controls

Prior to moving on to analysis of the results, it is important to verify that the controls are behaving as expected. If this is not the case, the quantitative results may not be accurate, and further troubleshooting may be necessary.

Ideally, none of the negative control wells should cross the threshold; although it is not uncommon to see the negative controls drift across the threshold during late cycles. If the negative controls are displaying sigmoid-shaped amplification curves, the fact that real amplification of the negative control is taking place would be indicated. This may be due to template contamination or excessive primer dimer formation.

Whether this will affect the Cts of the unknown samples will depend on the level of the signal in your negative controls. If the Cts of the negative control wells are ten cycles higher than the Cts of any of the unknown wells, it is safe to assume that these results are accurate. If the Cts in the negative control wells are within five cycles of any of the unknowns, this may call the validity of the results into question. Under these circumstances it may be necessary to troubleshoot the reaction to determine the source of signal in the negative control wells. The type of negative control well from which the signal was detected can provide an important indication of the source of the trouble. A shift in the No RT controls would indicate possible genomic DNA contamination. A shift in the NAC control wells could indicate probe degradation and a shift in the NTC wells may indicate primer dimer formation (when performing a SYBR Green I assay), or contamination. If the shift in the negative control wells is due to primer dimers, you can determine if the primer dimers are also forming in the unknown wells by looking at the dissociation curves.

If the positive control wells are not showing amplification, it will call into question whether any of the unknown wells that did not amplify are actually negative samples or whether this is due to non-specific failure of the PCR reaction (e.g., the presence of an amplification inhibitor). In this case, it may be necessary to troubleshoot the reaction conditions (e.g., different water and/or primer sources). The presence of PCR inhibitors in the template can also be identified by decreasing the amount of template used. If the Ct values tend to decrease or remain constant in the presence of lower amounts of template, this usually indicates the presence of an amplification inhibitor.

Replicate Agreement

If replicate samples were run, verify that the replicate wells are tightly grouped. If any well in a replicate set is an obviously anomalous point (e.g., the Ct is coming up very late/not at all, or there is excessive spiking) you should go back under Analysis Selection/Setup and assign this well its own replicate symbol or deselect it. This will prevent it from interfering with the calculations for the other replicate wells. Consistently poor replicate uniformity could indicate possible problems in the experimental setup and will definitely affect the accuracy of your results.

Standard Curve Quantification

After amplification given that both the standards and experimental samples are amplifying efficiently, the Ct's for each standard dilution can be determined and plotted against the initial template quantity. Sample Ct values can be used to estimate template quantity by comparing them to the standard curve. For this estimate to be accurate, the standard curve must be linear across the whole range of template concentrations in your assay and the measured efficiency of amplification near 100%.

A typical plate setup for a standard curve can be seen in Figure 18. The Ct values from each standard well will be used to create a standard curve. Figure 28 represents a typical standard curve constructed over three orders of magnitude (40 copies to 20,000 copies) on an Mx instrument.

Data from a standard curve run can be viewed in multiple formats including: Standard Curve, Initial Template Quantity, and Plate Sample Values.

All	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard REF 2.00e+004	Standard REF 2.00e+004	Standard REF 2.00e+004	Standard REF 1.00e+004	Standard REF 1.00e+004	Standard REF 1.00e+004	Standard REF 5.00e+003	Standard REF 5.00e+003	Standard REF 5.00e+003	Standard REF 2.50e+003	Standard REF 2.50e+003	Standard REF 2.50e+003
B	Standard REF 1.25e+003	Standard REF 1.25e+003	Standard REF 1.25e+003	Standard REF 6.25e+002	Standard REF 6.25e+002	Standard REF 6.25e+002	Standard REF 3.13e+002	Standard REF 3.13e+002	Standard REF 3.13e+002	Standard REF 1.57e+002	Standard REF 1.57e+002	Standard REF 1.57e+002
C	Standard REF 7.85e+001	Standard REF 7.85e+001	Standard REF 7.85e+001	Standard REF 3.93e+001	Standard REF 3.93e+001	Standard REF 3.93e+001	NTC REF FAM	NTC REF FAM	NTC REF FAM	Unknown REF FAM	Unknown REF FAM	Unknown REF FAM

FIGURE 18: INTRODUCCION TO QUANTITATIVE PCR - STRATAGENE®

Figure 18. Example of a standard curve plate setup. This two-fold dilution series would generate a 10-point standard curve in triplicate, from 20,000 copies down to about 40 copies.

In the standard curve view, as seen in Figure 19, the efficiency and linearity will automatically be displayed by the software using the equation:

$$X_n = X_0(1+E)^n$$

Where X_n = amplified target amount (target quantity at cycle n); X_O = starting quantity; E = efficiency of amplification; and n = number of cycles. When the efficiency is perfect (100% or 1), there is a perfect doubling of target amplicon every cycle; a 10-fold amplification should take 3.32 cycles ($2^{3.32} = 10$). In a plot of Ct versus the log of initial template, the slope should therefore be close to -3.32 (negative because a higher Ct means lower template amount).. Because of this relationship, you can calculate the efficiency directly from the slope using the equation below:

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

In experiments where a standard curve is run, the slope should be in the range of -3.10 to -3.59, which would correlate to a 90-110% efficiency range. The RSq value for the standard curve should be 0.985 or higher. R squared indicates how well the data points fit to a straight line indicating both the agreement between your replicates and the linear range of the assay. If points are dropping off the linear at one end of the standard curve, this would indicate those concentrations are outside the linear range of detection for that assay, and further assay optimization may be necessary to accurately quantify sample concentrations in that range. Adjusting the threshold may help improve the slope and R squared to a certain extent as well.

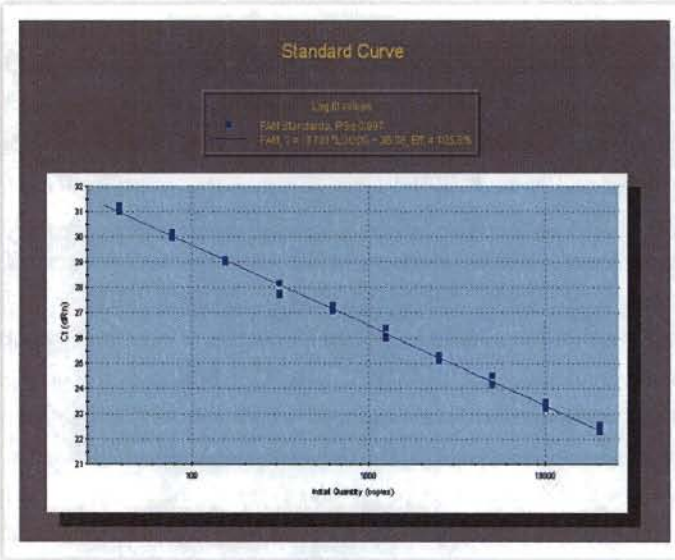


FIGURE 2: INTRODUCCION TO QUANTITATIVE PCR - STRATAGENE®

Figure 19. Standard curve demonstrating a two-fold dilution series from 20,000 to 40 copies. At each standard dilution a one-cycle change in Ct value is observed. This direct correlation between fold-decrease in standard concentration and increased Ct value demonstrates that the doubling efficiency of this assay is approximately 100%.

Real-Time PCR and its application for the detection of pathogens in seeds

Because planting seeds can serve as vectors for the survival and long distance dissemination of plant pathogens, seed health testing is critical for preventing the introduction of non-indigenous organisms, as well as for limiting losses from epidemics initiated by seedborne inoculum. Over the years, many conventional seed health assays have been employed and range widely in their levels of effectiveness, cost and ease of application. Seed assays that are widely employed include selective media, ELISA and seedling grow-out, and while they have been relied upon heavily; they have shortcomings that include the need for expertise in recognizing symptoms or morphological or physiological characteristics of the pathogen(s). Additionally, they generally take upwards of a week for completion and lack high levels of sensitivity. Finally, different approaches must be used to detect multiple pathogens in the same seed sample. These shortcomings highlight the need for an assay that is applicable for all pathogens in all seed types. Once such assay is **real time PCR**. Like conventional PCR, real time PCR involves the in vitro amplification of specific nucleotide sequences; however, it differs in that nucleic acid amplification is coupled to the release of a fluorescent reporter molecule (fluorophore) that can be monitored as it accumulates in the PCR reaction mixture. Reporter molecules can be fluorescent dyes that intercalate to the DNA double-strand (e.g., SYBR® GREEN or Ethidium bromide) or sequence-specific probes. Sequence-specific oligonucleotides labeled at the 5' end with reporter dyes (e.g. TaqMan®) and at the 3' end with a quencher molecule (e.g. TAMRA, BHQ1) hybridize with complementary sequences during the annealing step of PCR. In this configuration, when excited by light energy, the light fluorophore is absorbed by the quencher, hence no fluorescence is produced. During the extension step of PCR, the 5' to 3' exonuclease activity or Taq polymerase hydrolyzes the dual-labeled probe and releases the fluorophore from the quencher. When excited by light, the liberated fluorophore can then emit energy in a specific wavelength that can be detected by the real time PCR machine (Figure 20). With each subsequent PCR cycle, more fluorophore is released, resulting in an exponential increase in fluorescence that corresponds to increases in newly synthesized DNA amplicons.

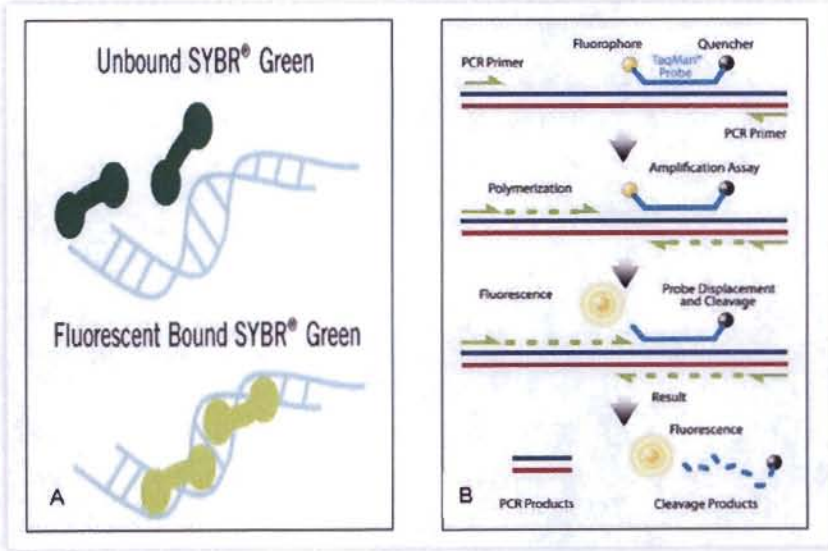


FIGURE 20: INTRODUCCION TO QUANTITATIVE PCR - STRATAGENE®

Figure 20. Reporter molecules used for real-time PCR. A) SYBR® Green I binds to double-stranded DNA, emitting 1,000-fold greater fluorescence than in the unbound state. B) Dual-labeled TaqMan® probe binds to the complementary target sequence and emits fluorescence when cleaved from the quencher molecule (2).

Real time PCR is simple, specific, rapid and highly sensitive. Additionally, the monitoring of DNA amplification does not require post-PCR processes such as gel electrophoresis and/or Southern hybridization. Real-time PCR results are interpreted by the cycle threshold value (Ct), which is the PCR cycle number at which fluorescence surpasses a pre-determined threshold value. Since this Ct value is negatively correlated to the starting amount of target template nucleic acid, quantification of seedborne pathogen inoculum is possible using this approach.

Another benefit of real-time PCR is the ability to easily facilitate multiplexing (simultaneous amplification and detection of different target nucleic acids). Currently, a range of reporter molecules are available (e.g., FAM, Texas Red, TAMRA etc.) that vary in their emission spectra (i.e. wavelength of light emitted after excitation). By combining fluorophores that have distinct light emission spectra, it is possible to simultaneously detect fluorescence associated with two independent PCR reactions in the same tube (Figure 21). Hence real time PCR can significantly improve the design and development multiplex PCR assays relative to conventional PCR. It should be noted, however; that all multiplex real time PCR assays should be empirically optimized as competition between reactions may lead to inhibition and false-negative results under certain reaction conditions.

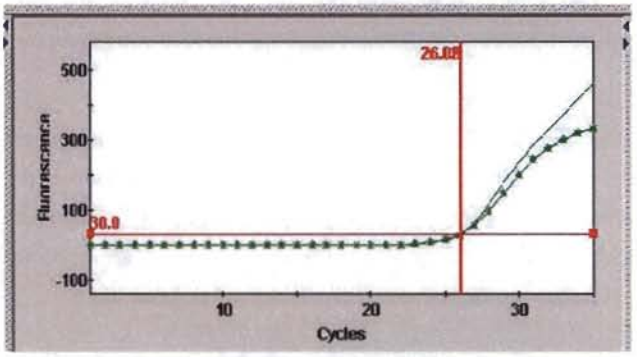


Figure 21. Results of multiplex time PCR for two target genes (i.e. two pathogen types). Two fluorescent signals are simultaneously detected/ amplified in the same reaction.

Because of the power of real time PCR, it is widely used for diagnostic purposes in many fields including clinical, veterinary and food microbiology (1, 2, 3). Real time PCR fulfills many of the requirements for effective seed health testing including speed, specificity and sensitivity. Thus, it has great potential to replace current seed health assays. However, to date, real time PCR-based seed health testing is under utilized in commercial seed testing labs. The objective of these exercises is to provide hands-on experience with conducting real time PCR and immuno magnetic separation with real time PCR for the important seedborne pathogen: *Acidovorax avenae* subsp. *citrulli* the causal agents of bacterial fruit blotch of cucurbits.

REFERENCES

1. Hein I, Klein D, Lehner A, Bubert A, Brandl E, and Wagner M. 2001. Detection and quantification of the *iap* gene of *Listeria monocytogenes* and *Listeria innocua* by a new real-time quantitative PCR assay. Res. Microbiol. 152: 37–46.
2. Introduction to quantitative PCR: Methods and Application Guide. 2007. Stratagene.
3. Kristiansen BE, Ask E, Jenkins A, Fermer C, Rådstrøm P, Skøld O. 1991. Rapid diagnosis of meningococcal meningitis by polymerase chain reaction. Lancet. 337:1568-9.
4. Valasek, Mark A. and Repa, Joyce, J. 2005. The power of real-time PCR. Adv. Physiol. Educ. 29:151-159.

Experiment and Chemistry Terms

Allele Discrimination (Real-Time) Real-time measurements using Ct to determine the genotype of a DNA sample. To achieve discrimination, two probes are used to identify the wild type and mutant alleles. A DNA sample genotype is determined by plotting the Ct value specific to the wild-type allele against Ct specific to the mutant allele. This can be performed in separate tubes or in multiplex if the different probes are each labeled with spectrally distinct dyes.

Allele Discrimination (Plate Read) Plate read measurements of fluorescence are used to determine the DNA sample genotype. To achieve discrimination, two probes labeled with two spectrally distinct dyes are used to identify the wild type and mutant alleles. Results are analyzed as follows: (1) if the fluorescence value of the unknown DNA sample is high for the wild type dye and low for the dye identifying the mutant, the sample is called wild type homozygote. (2) If the fluorescent value from the unknown DNA sample is high for the dye identifying the mutant and low for the wild type dye the sample is called mutant homozygote. (3) If the sample generates intermediate values for both dyes, it is called heterozygote.

Comparative Quantitation A QPCR analysis method that enables determination of relative gene expression compared to a calibrator (a single standard). This method is used to establish relative fold-increase in expression by assuming unchanging reaction efficiency. This method eliminates the requirement to include a standard curve with each reaction. Comparative quantitation can be applied to DNA and cDNA targets, and the most common application is the comparison of mRNA expression levels in treated versus untreated or normal versus diseased cells or tissue.

Dissociation Curve A melting curve protocol that reports the temperature on the X-axis, versus either fluorescence (R, Rn) or the first derivative of fluorescence [-R'(T), -Rn'(T)] on the Y-axis. The analysis is used to verify the reliability of results from SYBR Green I quantitative experiments. SYBR Green I fluorescence exhibits a large increase upon binding to double-stranded DNA, and this can be used both to generate amplification plots real-time during the amplification and to obtain thermal denaturation profiles of the complex nucleic acid mixtures generated during PCR amplification. Typically, two semi-discrete populations with different transition temperatures can be identified in the first derivative plots. Populations with a Tm of 80°C or higher correspond to the larger PCR products, and are usually assigned to a specific DNA product. DNA products displaying melting temperatures less than 75°C correspond to non- specific DNA products that are not necessarily homogeneous and may: contain multiple PCR product species.

Dynamic Range The range of fluorescence signal (from the lowest to the highest in the experiment) in which there is a direct linear relationship between actual fluorescence and reported signal. This range lies between the background noise on the lower end and the point where the detector starts to become saturated on the higher end. A wide dynamic range in a real-time system confers the ability to detect samples with high and low copy number in the same run.

Molecular Beacon Melting Curve After the Molecular Beacon is manufactured, the melting characteristics should be verified using a melt curve analysis protocol to determine the Molecular Beacon's target specificity, melting temperature (Tm), and appropriate annealing temperature for subsequent PCR experiments, The melt curve displays the Molecular Beacon fluorescence at various temperatures in the presence or absence of single-stranded

oligonucleotide target. For allele discrimination assays, the melting curve performed with the matched and the mismatched synthetic target defines the optimal temperature for assay discrimination performance.

Molecular Beacon Probes Hairpin-shaped fluorescence-labeled probes that can be used to monitor PCR product formation either during or after the amplification process. The free probe maintains the hairpin structure and causes quenching of the fluorophore. When the probe is annealed to target the fluorophore is separated from the quencher and fluorescence can be detected.

Plate-Read (Endpoint) Experiments A single measurement of the fluorescence taken at the completion of the amplification reaction. Results are generally recorded as either a positive or negative call on whether amplification occurred. Quantitation based on endpoint fluorescence is generally not as accurate as a real-time quantitative PCR assay.

Qualitative Detection Determination of the presence or absence of template of interest based on either Ct values or endpoint fluorescence.

Quantitative PCR Analysis Determination of either the starting concentration of a template of interest or the relative ratio of the quantity of a template in two different samples. This is based on either product measurement after the PCR reaction is complete or monitoring fluorescence intensity during the PCR reaction at each cycle in a closed-tube system. Methods for both RNA and DNA are available to determine mRNA signal levels and/or DNA gene quantification. S Quantitative PCR analysis software uses absolute standard curves, relative standard curves, or comparative methods for data analysis.

Quencher A compound used in QPCR experiments that absorbs the energy of the reporter dye in its excited state. The quencher can emit its own fluorescent signal (e.g. TAMRA) or emit no fluorescent signal (e.g. DABCYL, Black Hole Quencher).

Real-Time Experiments QPCR- experiments that monitor and report the accumulation of PCR product by measuring fluorescence intensity at each cycle while the amplification reaction progresses. Data are collected at the end of each melt/elongation cycle of the thermal cycling.

Reference Dye Dye used in real-time experiments for normalization of the fluorescence signal of the reporter fluorophore. The reference dye fluoresces at a constant level from cycle to cycle during the reaction, and if the reaction was aliquotted properly it will be at the same concentration in every sample. To normalize, the fluorescence signal of the reporter dye at each cycle is divided by the fluorescence signal of the reference dye in that tube at the same cycle, and normalized results are displayed as a ratio of the signal from the two dyes. ROX is commonly used as a reference dye.

Reporter Dye The fluorescent dye used to monitor PCR product accumulation in a QPCR experiment. This can be attached to a probe (such as with TaqMan or Molecular Beacons) or free in solution (such as SYBR Green I). Also known as the fluorophore.

Sensitivity of Detection The level at which a given assay is able to detect low copy numbers of the product of interest. This is important when working with samples that have low expression levels.

TaqMan Probes Linear FRET fluorescence-labeled probes used to monitor PCR product formation either during or after the amplification process. As the DNA polymerase extends the upstream primers and encounters the downstream probe, the 5' to 3' nuclease activity of the polymerase cleaves the probe. Following cleavage, the reporter fluorophore is released into the reaction solution and fluorescence is detected.

Exercise I:

PCR amplification of DNA from *Acidovorax avenae* subsp. *citrulli*

-in water and watermelon seed wash-

A. SAMPLES: Acidovorax avenae subsp. citrulli CELL SUSPENSION

1. Six samples of serially diluted Aac in water and watermelon seed wash. Plus a positive and negative control
   a. 10^6 CFU/ml. Aac in water and watermelon seed wash
   b. 10^5 CFU/ml. Aac in water and watermelon seed wash
   c. 10^4 CFU/ml. Aac in water and watermelon seed wash

B. PREPARING REAL TIME PCR SAMPLES

2. Allow all PCR reagents melt completely on crushed ice. Place PCR tubes on crushed ice.
3. According to the ratios listed below, calculate the volume of each reagent, and add them into a sterile microcentrifuge tube. Briefly, vortex to mix the PCR mastermix and then centrifuge the tube.

PCR regents			# of reacciones		Volume of each reagent (µL)
Bioline Master Mix	6.3	X	10	=	
AACF3 (25 µM)	0.3	X	10	=	
AACR2 (25 µM)	0.3	X	10	=	
AAC Probe2 (10 µM)	0.5	X	10	=	
Sterile PCR-grade water	12.6	X	10	=	
		X	10	=	

4. Transfer 20 µl of this PCR mastermix in Step 2 to each PCR tube.
5. Add 5 µl of DNA sample or sterile water to each corresponding tube.
6. Place PCR tubes in real time PCR machine
7. Click **Start Run** to start the new run.

For the serial dilution assay use the following real time PCR conditions listed below.

Step 1 95°C 180 sec optics off
Step 2 95°C 15 sec optics off
Step 3 60°C 40 sec optics on

Repeat steps 2 & 3 35 times.

To establish a baseline threshold, run PCR using the tenfold serial dilutions of Aac cultures provided and an additional negative control (water). Based on the results of this serial dilution adjust your threshold such that the baseline is above the negative control fluorescence line but below that for the lowest positive check.

C. RESULTS

A. avenae subsp. citrulli cell suspension (CFU/ml)	PCR results	
	Serial dilutions in water (Ct value)	Serial dilutions in watermelon seedwash (Ct value)
10^6		
10^5		
10^4		
Positive control (10^6 Aac)		
Negative control (DDW)		

Exercise II:

IMS-PCR with *A. avenae* subsp. *citrulli* cell supension

A. SAMPLES: *Acidovorax avenae* subsp. *citrulli* CELL SUSPENSION in seed wash

1. Two samples of serially diluted Aac in watermelon seed wash. Plus a positive and negative control

   a. 10^6 CFU/ml. Aac in watermelon seed wash

   b. 10^5 CFU/ml. Aac in watermelon seed wash

B. IMS of *Acidovorax avenae* subsp. *citrulli*

2. Add 250 µl anti-Aac coated immunomagnetic beads (IMBs) (Quick Beads, Envirologix Inc.) (supplied) into each sample tube (Figure 1).

Figure 1. Gently add coated IMBs to sample.

3. Incubate samples for 1 h on an end-over-end sample mixer with gentle agitation (30 rpm). (Fig. 2)

Figure 2. Example of an end-over-end mixer.

4. Remove tubes from sample mixer and place in magnetic particle concentrator (MPC) (Figure 3).

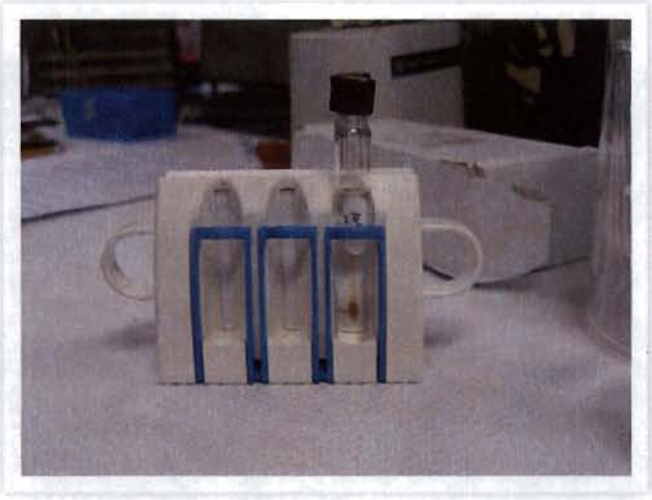


Figure 3. Example of an MPC

5. Use the MPC to rinse IMBs by removing buffer from each tube using a sterile Pasteur pipette and adding 8 ml of PBS-BSA. Repeat 3 times.

6. Rinse IMBs a 4th time with 8ml of sterile deionized water. Resuspend IMBs in 500 µl water and transfer to a sterile microcentrifuge tube.

7. Place sample tube in MPC and remove excess water without disturbing IMBs. (Figure 4)

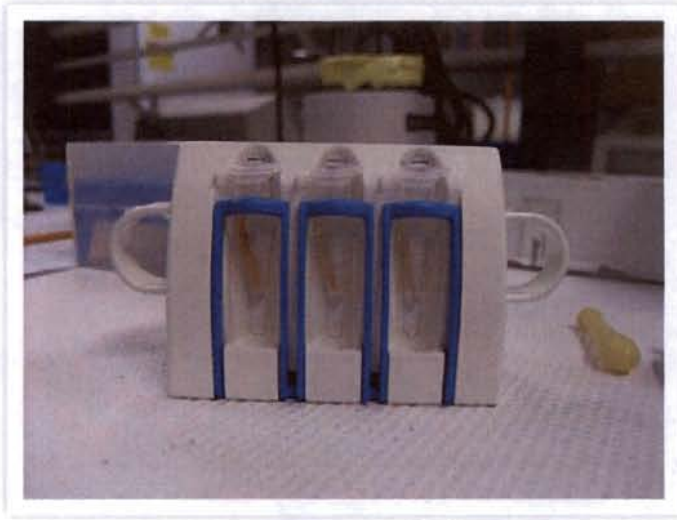


Figure 4. Concentrate samples in microcentrifuge tube

8. Add 20 µl of sterile deionized water.

9. Incubate IMBs (with attached bacteria) at 100°C for 10 min.

10. Centrifuge samples at 13,000 rpm for 15 seconds to collect IMBs (Figure 5). Use DNA released by lysis as template for PCR. **Do not transfer IMBs into the PCR reaction!!!** PCR can be run immediately or at a later date. If PCR will be run later, DNA samples must be frozen.

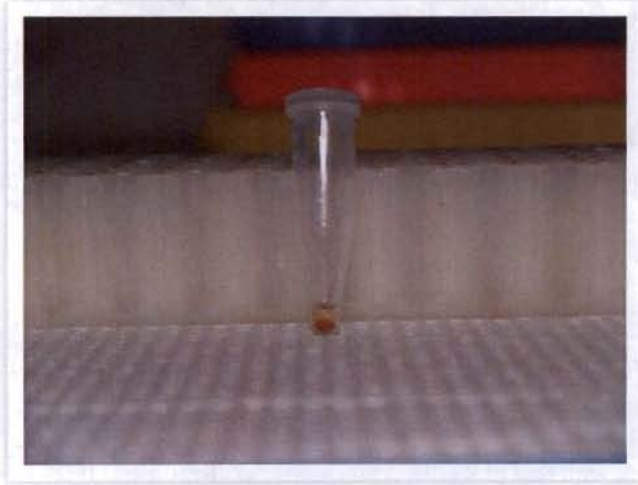


Figure 5. IMBs after cells have been lysed by boiling

C. PREPARING REAL TIME PCR SAMPLES

11. Allow all PCR reagents to melt completely on crushed ice. Place four PCR tubes on crushed ice.

12. According to the ratios listed below, calculate the volume of each reagent, and add them into a sterile microcentrifuge tube. Briefly, vortex to mix the PCR mastermix and then centrifuge the tube.

PCR Reagent			# of reacciones		Volume of each reagent (µL)
BioRad Master Mix	12.5	x	6	=	
AACF3 (25 µM)	0.3	x	6	=	
AACR2 (25 µM)	0.3	x	6	=	
AAC Probe2 (10 µM)	0.5	x	6	=	
Sterile PCR-grade water	6.4	x	6	=	
		x	6	=	

13. Transfer 20 µl of this PCR mastermix in Step 2 to each PCR tube.

14. Add 5 µl of DNA sample or sterile water to each corresponding tube.

15. Centrifuge briefly and transfer contents in to PCR plate. Place plate in real time PCR machine

16. Click **Start Run** to start the new run.

For the serial dilution assay use the following real time PCR conditions listed below.

Step 1 95°C 180 sec optics off

Step 2 95°C 15 sec optics off

Step 3 60°C 40 sec optics on

Repeat steps 2 & 3 35 times.

To establish a baseline threshold, run PCR using the tenfold serial dilutions of Aac cultures provided and an additional negative control (water). Based on the results of this serial dilution adjust your threshold such that the baseline is above the negative control fluorescence line but below that for the lowest positive check.

D. RESULTS

A. avenae subsp. citrullicell suspension (CFU/ml)		CT value
IMS samples	105	
	104	
PCR positive control	106	
PCR negative control	DDW	

Exercise III:

Detection of *A. avenae* subsp. *citrulli* in cucurbit seeds using IMS PCR

A. BACTERIAL EXTRACTION FROM SEEDS

1. Place sample (5,000 seeds) into flasks (Figure 1).

Positive control	5,000 clean watermelon seeds with infested seeds
Negative control	5,000 clean watermelon seeds

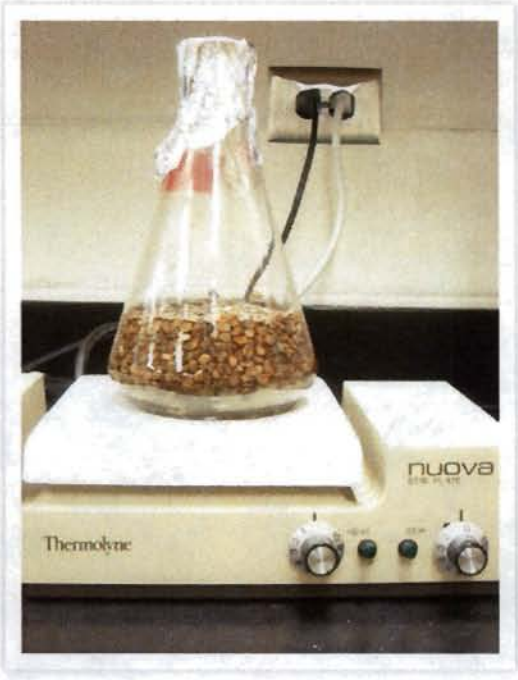


Figure 1. 5,000 seeds in 1 L flask

2. Add 350 ml of sterile 0.1 M phosphate buffered saline (PBS) (Appendix A1).

3. Shake at 250 rpm for 1hr.

4. Pass seed wash through four layers of cheesecloth. Use a sterile funnel to collect the filtrate in a sterile 500 ml flask (Figure 2).

Figure 2. Seeds are collected using 4 layers of cheesecloth

5. Measure the volume of seed wash and add 1/20 of the volume of pectinae (e.g. if the vol. of seed wash is 200 ml, add 10 ml of pectinase)

6. Shake samples with for 1 hr

7. Pour seed wash through Whatman #1 filter paper into a sterile 1 L flask. Filter 30-60 minutes (Figure 3)

Figure 3. Filtration of seed wash.

8. Collect approximately 50 ml of seed wash and centrifuge for 15 minutes at 9,600 rpm (Figures 4 and 5).

Figure 4. Seed wash after filtration

Figure 5. Example of centrifuge

9. Resuspend pellet in 6 ml 0.1M PBS with bovine serum albumin (PBS-BSA) (Appendix A2).

B. IMS of *Acidovorax avenae* subsp. *citrulli*

10. Place 6 ml of each seed extract into a sterile 9 ml glass tube with screw cap.

11. Add 250 µl anti-Aac coated immunomagnetic beads (IMBs) (Quick Beads, Envirologix Inc.) (supplied) into each sample tube (Figure 6).

12. Additionally, add 250 µl anti-Aac coated IMBs to a positive control sample with 10^4 CFU/ml (supplied)

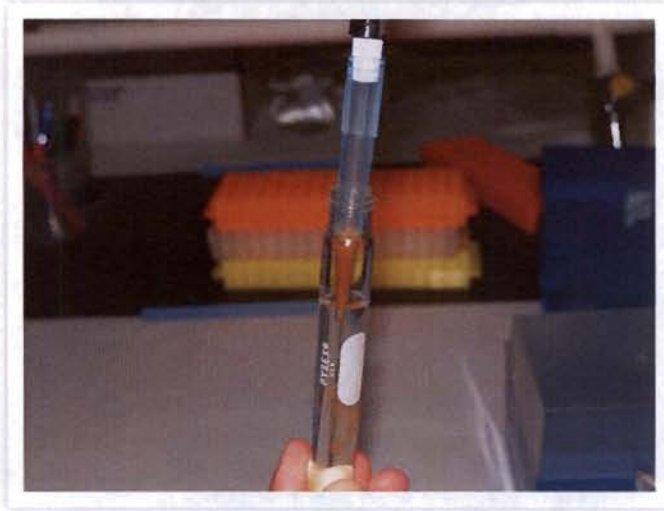


Figure 6. Gently add coated IMBs to sample

13. Incubate samples for 1 h on an end-over-end sample mixer with gentle agitation (30 rpm). (Figure 7)

Figure 7. Example of an end-over-end mixer.

14. Remove tubes Remove tubes from sample mixer and place in magnetic particle concentrator (MPC) (Figure 8).

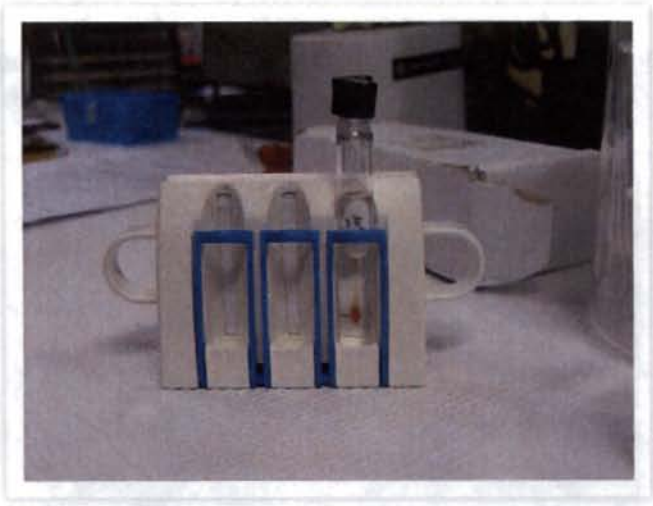


Figure 8. Example of an MPC

15. Use the MPC to rinse IMBs by removing buffer from each tube using a sterile Pasteur pipette and adding 8 ml of PBS-BSA. Repeat 3 times.

16. Rinse IMBs a 4th time with 8ml of sterile deionized water. Resuspend IMBs in 500 µl water and transfer to a sterile microcentrifuge tube.

17. Place sample tube in MPC and remove excess water without disturbing IMBs. (Figure 9)

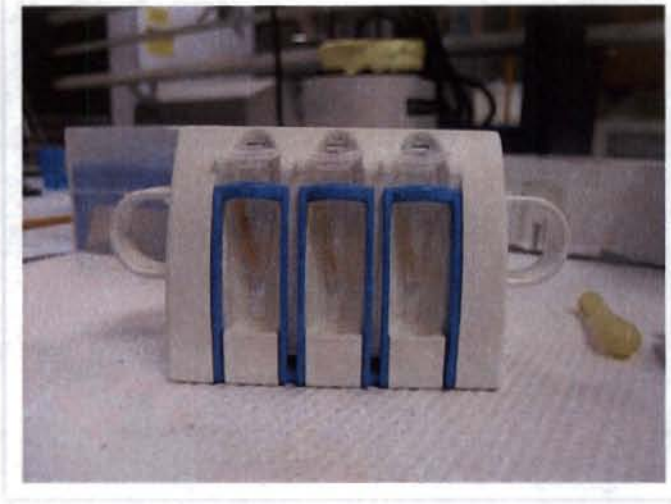


Figure 9. Concentrate samples in microcentrifuge tube

18. Add 20 µl of sterile deionized water.

19. Incubate IMBs (with attached bacteria) at 100°C for 10 minutes.

20. Centrifuge samples at 13,000 rpm for 15 seconds to collect IMBs (Figure 10). Use DNA released by lysis as template for PCR. **Do not transfer IMBs into the PCR reaction!!!** PCR can be run immediately or at a later date. If PCR will be run later, DNA samples must be frozen.

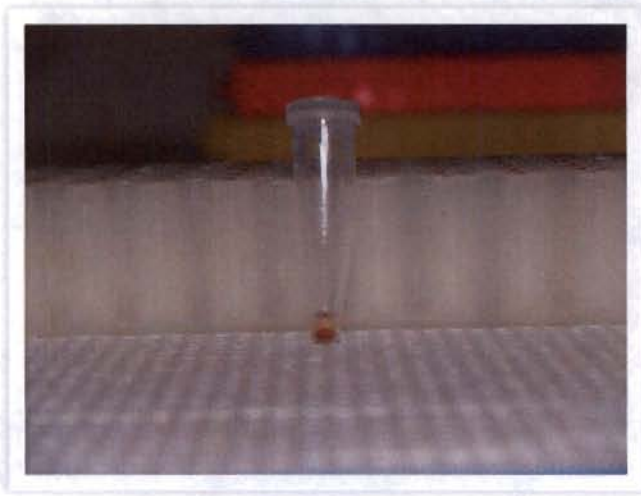


Figure 10. IMBs after cells have been lysed by boiling

C. PREPARING REAL TIME PCR SAMPLES

21. Allow all PCR reagents melt completely on crushed ice. Place five PCR tubes on crushed ice.

22. According to the ratios listed below, calculate the volume of each reagent, and add them into a sterile microcentrifuge tube. Briefly, vortex to mix the PCR mastermix and then centrifuge the tube.

Reactivo de PCR			# de reacciones		Volumen de cada reactivo (µl)
BioRad Master Mix	12.5	X	6	=	
Primer1 (50 µM)	0.25	X	6	=	
Primer 2 (50 µM)	0.25	X	6	=	
AAC Probe 5 (10 µM)	0.125	X	6	=	
Sterile PCR-grade wáter	6.9	X	6	=	
		X	6	=	

23. Transfer 20 µl of this PCR mastermix in Step 2 to each PCR tube.

24. Add 5 µl of DNA sample or sterile water to each corresponding tube.

25. Centrifuge briefly and transfer contents into a PCR plate. Place plate in real time PCR machine

26. Click **Start Run** to start the new run.

For the seed sample assay use the following real time PCR conditions listed below.

Step 1 95°C 180 sec optics off

Step 2 95°C 15 sec optics off

Step 3 62°C 20 sec optics on

Repeat steps 2 & 3 40 times.

To establish a baseline threshold, run PCR using the tenfold serial dilutions of Aac cultures provided and an additional negative control (water). Based on the results of this serial dilution adjust your threshold such that the baseline is above the negative control fluorescence line but below that for the lowest positive check.

C. RESULTS

Samples		Ct Values
Positive	5,000 clean watermelon seeds with infested seeds	
Negative	5,000 clean watermelon seeds	
IMS positive	10^4 CFU/ml A. avenae subsp. citrulli culture	
PCR positive	10^6 CFU/ml A. avenae subsp. citrulli culture	
PCR negative	DDW	

Practica I:

Amplificación de *Acidovorax avenae* subsp. *citrulli*

-en agua y lavado de semillas-

A. Muestras: ***Acidovorax avenae*** **subsp.** ***citrulli***

a. 10^6 CFU/ml. Aac en agua y lavado de semillas

b. 10^5 CFU/ml. Aac en agua y lavado de semillas

c. 10^4 CFU/ml. Aac en agua y lavado de semillas

B. Preparar los reactivos para la PCR

1. Permita que todos los reactivos de PCR se descongelen sobre el hielo.
2. De acuerdo al número de reacciones, multiplique la cantidad de reactivos que debe utilizar y adiciónelo al tubo marcado como **"Bioline".**

Reactivo de PCR			# of reacciones		Volumen de cada reactivo (µl)
AACF3 (25 µM)	0.3	X	10	=	
AACR2 (25 µM)	0.3	X	10	=	
AAC Probe2 (10 µM)	0.5	X	10	=	
Sterile PCR-grade water	12.6	X	10	=	
		X	10	=	

3. Transfiera 20 µl de este coctel a cada tubo para el PCR.
4. Añada 5 µl de muestra o agua esterilizada a cada tubo correspondiente.
5. Ponga los tubos de PCR en la máquina de Real Time PCR
6. Oprima **Start Run** para empezar una nueva corrida.

Practica II:

IMS-PCR con suspensión de *A. avenae* subsp. *citrulli*

A. Muestras: Suspensión de *Acidovorax avenae* subsp. *citrulli* en lavado de semillas

a. 10^6 CFU/ml. Aac en lavado de semillas

b. 10^5 CFU/ml. Aac en lavado de semillas

B. IMS de *Acidovorax avenae* subsp. *citrulli*

1. Añada 250 µl de anti-Aac immunomagnetic beads (IMBs) (Quick Beads, Envirologix Inc.) a cada tubo.
2. Incube las muestras por 1 hora en un balancín.
3. Retire los tubos del balancín y póngalos en el magnetic particle concentrator (MPC).
4. Utilice el MPC para enjuagar los IMBs removiendo la solución tampón de cada tubo con una pipeta Pasteur y añadiendo 8 ml de PBS-BSA. Repita 3 veces.
5. Enjuague los IMBs por cuarta vez utilizando 8ml de agua esterilizada. Resuspenda los IMBs en 500 µl de agua y transfiera la muestra a un tubo de 1.5 ml.
6. Ponga el tubo con la muestra en el MPC, remueva el agua en exceso sin remover los IMBs.
7. Añada 20 µl de agua esterilizada.
8. Incube los IMBs a 100°C por 10 min.
9. Brevemente centrifugue las muestras para colectar los IMBs. Utilice el ADN producto de la lisis como muestra para el PCR. **No transfiera los IMBs al tubo de PCR!.** El PCR se puede correr inmediatamente o conservar las muestras congeladas.

B. Preparar los reactivos para el PCR

1. Permita que todos los reactivos de PCR se descongelen sobre el hielo
2. De acuerdo al número de reacciones multiplique la cantidad de reactivos que debe utilizar

Reactivo de PCR			# of reacciones		Volumen de cada reactivo (µl)
BioRad Master Mix	12.5	X	6	=	
AACF3 (25 µM)	0.3	X	6	=	
AACR2 (25 µM)	0.3	X	6	=	
AAC Probe2 (10 µM)	0.5	X	6	=	
Sterile PCR-grade water	6.4	x	6	=	
		x	6	=	

3. Transfiera 20 µl de este cóctel a cada tubo para el PCR.
4. Añada 5 µl de muestra o agua esterilizada al tubo correspondiente.
5. Ponga los tubos de PCR en la máquina de Real Time PCR.
6. Oprima **Start Run** para empezar una nueva corrida

Practica III:

Detección de *A. avenae* subsp. *citrulli* en semillas de sandia utilizando IMS-PCR

A. Extracción de bacteria a partir de las semillas

1. Ponga cada muestra (5,000 semillas) dentro de un flask de un litro.
2. Añada 350 ml de 0.1 M phosphate buffered saline (PBS).
3. Agite por 1 hora.
4. Pase el lavado de semillas por cuatro capas de gasa utilizando un flask de 500 ml y un embudo.
5. Mida el volumen del lavado de semillas y añada pectinasa a una proporción 1/20 del volumen total de la muestra. (e.g. si el volume del lavado de semillas es 200 ml, añada 10 ml of pectinasa)
6. Agite por 1 hr.
7. Pase el lavado de semillas a través de papel filtro, utilizando un flask y un embudo.
8. Transfiera aproximadamente 40 ml del lavado de semilla un tubo de centrifugación y centrifugue por 15 minutos a 9,600 rpm
9. Resuspenda el precipitado en 6 ml de 0.1M PBS más bovine serum albumin (PBS-BSA).

B. IMS of *Acidovorax avenae* subsp. *citrulli*

1. Transfiera 6 ml de cada muestra a un tubo de vidrio esterilizado de 9 ml.
2. Añada 250 µl de anti-Aac immunomagnetic beads (IMBs) (Quick Beads, Envirologix Inc.) a cada tubo.
3. Adicionalmente, añada 250 µl de anti-Aac coated IMBs a un control positivo con 10^4 CFU/ml.
4. Incube las muestras por 1 hora en un balancín.
5. Retire los tubos del balancín y póngalos en el magnetic particle concentrator (MPC).
6. Utilice el MPC para lavar los IMBs, removiendo la solución tampón de cada tubo con una pipeta Pasteur y añadiendo 8 ml de PBS-BSA. Repita 3 veces.
7. Lave los IMBs por cuarta vez utilizando 8ml de agua esterilizada. Resuspenda los IMBs en 500 µl de agua y transfiera la muestra a un tubo de 1.5 ml.
8. Ponga el tubo con la muestra en el MPC, remueva el agua en exceso sin remover ni tocar los IMBs.

9. Añada 20 µl de agua esterilizada.

10. Incube los IMBs a 100°C por 10 min.

11. Brevemente centrifugue las muestras para colectar los IMBs. Utilice el ADN producto de la lisis como muestra para el PCR. **No transfiera los IMBs al tubo de PCR! .** El PCR se puede correr inmediatamente o conservar las muestras congeladas.

C. Preparar los reactivos para el PCR

1. Permita que todos los reactivos de PCR se descongelen sobre el hielo

2. De acuerdo al número de reacciones multiplique la cantidad de reactivos que debe utilizar.

Reactivo de PCR			# de reacciones		Volumen de cada reactivo (µl)
BioRad Master Mix	12.5	X	6	=	
Primer1 (50 µM)	0.25	X	6	=	
Primer 2 (50 µM)	0.25	X	6	=	
AAC Probe 5 (10 µM)	0.125	X	6	=	
Sterile PCR-grade water	6.9	X	6	=	
		X	6	=	

3. Transfiera 20 µl de este cóctel a cada tubo para el PCR.

4. Añada 5 µl de muestra o agua esterilizada al tubo correspondiente.

5. Ponga los tubos de PCR en la máquina de RealTime PCR.

6. Oprima **Start Run** para empezar una nueva corrida

Practica IV:

Detección de un Fitoplasma del subgrupo 16srIII-L asociado con Cuero de Sapo

Metodología para Extracción de ADN Total

Método según Wizard Genomic DNA Purification Kit (Promega, Madison, WI)

1. Tomar 40 mg de tejido y macerar usando bolsa plástica con malla.
2. Adicione 600 µL de Nuclei Lysis Solution.
3. Incubar a 65 °C por 15 minutos, recupere la mayor cantidad de la solución (100 µL – 200 µL aproximadamente) con el macerado en la bolsa y transfiriendo a tubo de 1.5 mL.
4. Adicione 3 µL de RNase Solution y mezcle agitando el tubo 2-5 veces. Incube a 37 °C por 15 minutos y luego deje la muestra a temperatura ambiente por 5 minutos.
5. Adicione 200 µL de Protein Precipitation Solution y homogenice en vortex a alta velocidad por 20 segundos.
6. Centrifugue por 3 minutos a 14000 r.p.m.
7. Cuidadosamente remueva el sobrenadante el cual contiene el DNA (deje el precipitado de proteínas en el tubo) y transfiéralo a un tubo de 1.5 mL con 600 µL de isopropanol a temperatura ambiente.
8. Mezcle fuertemente la solución por inversión.
9. Centrifugue la mezcla a 14000 r.p.m por 1 minuto a temperatura ambiente.
10. Elimine el sobrenadante con cuidado. Adicione 600 µL de etanol al 70% a temperatura ambiente e invierta el tubo varias veces para limpiar el ADN. Centrifugue a 14000 r.p.m por 1 minuto a temperatura ambiente.
11. Elimine el etanol aspirándolo con una micropipeta o invirtiendo el tubo con cuidado de no perder el pellet. Evite aspirar el precipitado con la pipeta.
12. Invierta el tubo sobre una toalla de papel absorbente durante 15 minutos para secar el precipitado de ADN.
13. Adicione 100 µL de ADN Rehydration Solution y resuspenda el precipitado o pellet. También se puede incubar toda la noche a 4 °C. Almacenar el ADN a 2-8 °C

Metodología para Extracción de ADN Total

Método buffer fosfato

1. Tomar 0.1 g de tejido adicionar 1 ml de buffer fosfato y macerar usando bolsas plasticas (plastic bags with a heavy net.)
2. Tomar de la bolsa 20 µL del macerado y realizar diluciones seriadas hasta $1x10^{-8}$.
3. Incubar a 96 °C por 4 minutos
4. Colocar en hielo por 2 minutos.

   Tomar 3 µL cada dilución para QPCR.

La sonda y cebadores para la detección del fitoplasma 16SrIII-L asociado con el cuero de sapo de la yuca, se diseñaron basados en el gen *rp* que codifica para proteínas ribosomales. Este gen es utilizado para la clasificación taxonómica de fitoplasmas, debido a que es altamente conservado y tiene una menor numero de características similares con bacterias gram positivas y entre los diferentes *Cadidatus fitoplasma*, lo que otorga una mayor especificidad a la sonda y cebadores diseñados (Figura 1y Tabla 1).

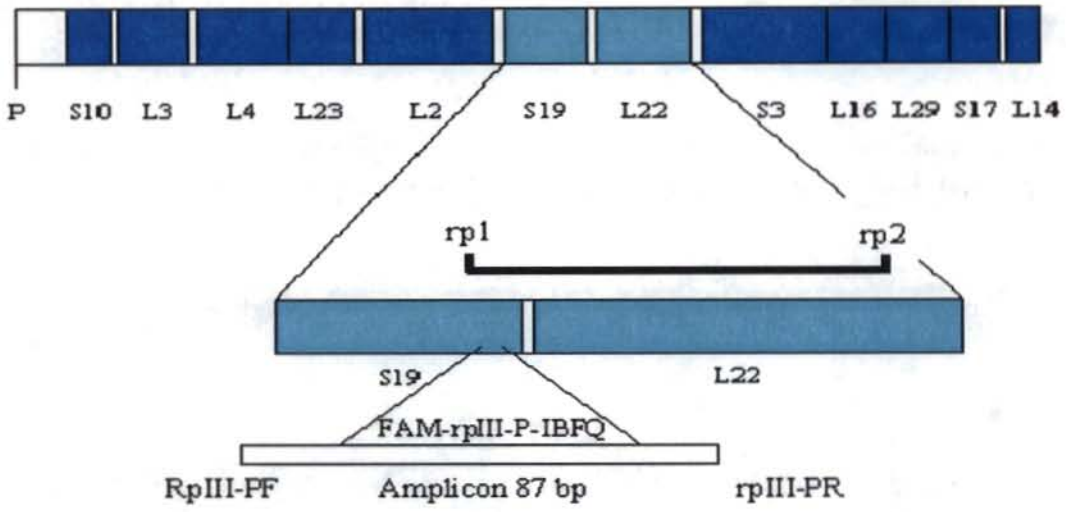


Figura 1. Ubicación del amplicon 87 bp dentro del gen *rp*.

Tabla 1. Cebadores y sonda utilizados para la amplificación por PCR en tiempo real del fitoplasma 16SrIII-L asociado con el cuero de sapo de la yuca.

Nombre	Tm	Tamaño de amplicon (en pb)	Gen	Fluorocromo
rpIII-PF	56.3	87	Rp	....
rpIII-PR	57.8	....	....	....
rpIII-P	64.9	....	....	FAM/IBFQ

Procedimiento

La totalidad del procedimiento para las reacciones de PCR en tiempo real se debe hacer en la cámara de flujo laminar y los reactivos deben mantenerse en hielo.

Las condiciones a aplicar (Tabla 2) para cada reacción PCR con un volumen final de 25µl, son las siguientes: Buffer *Taq* (BIOLASE™ DNA Polymerase, BIOLINE) 10xNH4 (160mM (NH4)2SO4, 670Tris-HCl pH 8.0, 0.1% Tween-20), a una concentración final de 1X/µL; 0.2 mM de cada uno de los dNTPs, 30 nM Reference dye, 200 nM de sonda rpIII-p, 600 nM de cebador rpIII-PF, 200 nM de cebador rpIII-PR, 1.5 U *Taq* Polimerasa, 3 mM de MgCl2, 3

µL de ADN total, agua HPLC esterilizada a través de un filtro de membrana de 0.22 µm de tamaño de poro (MILLEX®-GV; Millipore Products Division, Bedford, MA) y por autoclave.

Preparar la mezcla indicada en la Tabla 2, para esto calcule el volumen total teniendo en cuenta el número de muestras a procesar, incluyendo un control negativo que DEBE ser agregado de último y al menos un control positivo de una muestra conocida, dando el margen de una muestra adicional, que le permita corregir el error de pipeteo. Adicione cada componente en el orden indicado. El cóctel así preparado se distribuye en tubos para QPCR agregando a cada uno un volumen de 22 µl, luego incube las muestras para su amplificación por PCR en tiempo real, en un equipo Stratagene's Mx 3005P programado con el perfil de amplificación indicado en la Tabla 3.

Tabla 2. Concentraciones y volúmenes de componentes para una reacción de PCR en tiempo real para amplificación de una región del gen *rp* asociada con el fitoplasma 16SrIII-L asociado con el cuero de sapo de la yuca.

Reactivo	Concentración inicial	Concentración final	Reacción x 1 muestra (µL)	# de reacciónes		Volumen de cada reactivo (µL)
H2O destilada estéril	-	-	12.3	X	11	
Sonda rpIII-P	5000 nM	200nM	1	X	11	
Cebador rpIII-PF	10 µM	600nM	1.5	X	11	
Cebador rpIII-PR	10 µM	200nM	0.5	X	11	
ADN	-	-	3			
**Volumen Final			25 µL			

*Bioline (Nelson Royeiro & Cia)

** De acuerdo al número de reacciones, multiplique la cantidad de reactivos que debe utilizar y adiciónelo al tubo marcado como **"Bioline"**.

Tabla 3. Condiciones de amplificación del ADN para PCR en tiempo real de una región del gen *rp* del fitoplasma 16SrIII-L asociado con el cuero de sapo de la yuca.

Temperatura (°C)	Tiempo	No de Ciclos
95	10 min	1
95	30 seg	
60	1 min	40

Práctica V:

Detección de *Ralstonia solanacearum* causante del moko del plátano mediante PCR en Tiempo Real

La bacteria *Ralstonia solanacearum* es el agente causante de la marchitez bacteriana del plátano, también conocida como moko, enfermedad que limita gravemente la producción de plátano. El patógeno se caracteriza por su agresividad, facilidad de dispersión y alta variabilidad genética, causando importantes pérdidas económicas.

Existen diferentes métodos empleados para la detección de la bacteria en tejido de planta y suelo. Para la detección de este patógeno, principalmente en suelo, se utiliza la técnica de BIO-PCR, la prueba de hipersensibilidad en tabaco y pruebas bioquímicas para la identificación de biovares.

La técnica de BIO – PCR consiste en el aislamiento de colonias en medio semi- selectivo Sudáfrica (SMSA) (Denny y Hayward, 2001) y la amplificación con el cebador específico OLI1, junto con el cebador no específico Y2. La prueba de hipersensibilidad del tabaco consiste en inocular plantas tabaco con las bacterias aisladas de tejido enfermo. Esta metodología permite identificar cepas de la raza 2, patógenas para el plátano (Lozano y Sequeira, 1970). Un tercer método consiste en caracterizar biovares a través de pruebas bioquímicas (Denny y Hayward, 2001).

Sin embargo, estas metodologías presentan baja sensibilidad, especificidad y demanda de tiempo para el diagnóstico de la enfermedad. La detección e identificación de *R. solanacearum* mediante qPCR permite la detección en menor tiempo sin afectar la sensibilidad y especificidad, adicionalmente se podrá cuantificar la cantidad aproximada de células bacterianas, cualidades que optimizan la detección temprana del patógeno.

Recolección y conservación de muestras

El material a colectar debe estar conformado por tejidos jóvenes que presenten los síntomas típicos de la enfermedad, como seudotallo, peciolo, raíz y fruto. Los tejidos deben estar en buen estado, preferiblemente con síntomas tempranos e intermedios de la enfermedad y no presentar necrosis avanzada o deterioro del tejido.

En el momento de la colecta, el tejido seleccionado se envuelve en toallas de papel desechables o en papel kraff, luego éste se introduce en una bolsa plástica y se almacena en nevera sellada de icopor a ±10°C, preferiblemente con hielo. Estas condiciones deben ser mantenidas hasta el momento del análisis; si la muestra está húmeda al momento de tomarla, primero se seca al aire y posteriormente se envuelve como se mencionó anteriormente, con el fin de evitar el deterioro de ésta, lo que facilita la detección del patógeno. Bajo condiciones de laboratorio, las muestras pueden permanecerá a 4°C por máximo dos semanas, pero preferiblemente deben ser procesadas inmediatamente.

Diseño de la sonda

La sonda TaqMan® específica para la detección de *R. solanacearum* en musáceas se diseño a partir de cepas aisladas en Colombia y con base en la clasificación reportada por Fegan y Prior (2005), según la cual corresponde a las cepas de moko (filotipo II - sequevar 4). La asociación con la región ITS se realizó mediante correlación de patogenicidad de las

cepas y ausencia o presencia de fragmentos amplificados de sequevar 4. Con la región identificada, se utilizó el programa Primer Express ® software v. 2.0 para el diseño de la sonda TaqMan ® y los primers forward y reverse.

Referencias

Hayward, A. C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annual Review of Phytophatology. 29: 64-87.

Fegan M. and Prior P. 2005. How complex is the "*Ralstonia solanacearum* species complex". In 'Bacterial Wilt: The Disease and the *Ralstonia solanacearum* species complex'. (Eds C. Allen, P. Prior, A. C. Hayward) pp. 449-461. (APS Press: St. Paul).

Procedimiento

A. Materiales

- Tubos o tiras de tubos para qPCR.
- Centrifuga.
- Equipo para Real Time PCR (Light cycler machine).
- H_2O grado HPLC estéril y libres de DNAsas.
- Taqman PCR Master Mix (se debe preparar).
- Micropipetas y puntas estériles con filtro.
- DNA away.

B. Detección directa a partir de tejido

1. Macerar 0.05 gr de tejido fresco de seudotallo con síntomas de la enfermedad en bolsa plástica con malla, adicionando 0.45 ml de buffer fosfato pH 7.2, la suspensión se lleva a 96°C en baño maría durante cuatro minutos, seguido de incubación en hielo por dos minutos. El mismo procedimiento se realiza con tejido fresco sano.

2. La suspensión se trasfiere a un tubo de 1.5 ml y a partir de este se realizan diluciones seriadas en base 10 hasta 10^{-4}, luego de cada dilución se toma el volumen requerido para la reacción qPCR.

C. Detección directa utilizando el Wizard Genomic DNA Purification Kit (Promega®)

Seguir protocolo según Pag 61.

D. Real Time PCR

1. Inicialmente se calculará las cantidades de cada reactivo para conformar el cóctel (Master mix) para 2 muestras de plantas con moko, uno control positivo y dos control negativos. El volumen final es para 8 reacciones (Tabla 1).

Tabla 1. Concentraciones y volúmenes de los reactivos que se utilizan para una reacción y/o 8 reacciones de qPCR

Reactivos	Concentración Stock	Concentración Final	Volumen para 1 reacción (µl)	Volumen para 8 reacciónes (µl)
Agua PLC	-	-	12.1	x 8
dNTP's	2 mM	200 µM	2.5	x 8
PCR Buffer*	10 X	1 X	2.5	x 8
Mus 20F	10 µM	300 nM	0.75	x 8
Mus 20PR	10 µM	300 nM	0.75	x 8
Sonda Mus 20P	5 µM	120 nM	0.6	x 8
$MgCl^2$*	25 mM	1.5 mM	1.5	x 8
Polimerasa*	5 U/µl	1.5 U/µl	0.3	x 8
Volumen final			21	

*Bioline (Nelson Royeiro & Cia)

2. Una vez realizados los cálculos se preparará el cóctel en un vial de 1 ml y se van mezclando los reactivos a medida que se adicionan. Este paso se deben mantener las condiciones de asepsia y esterilidad en cámara de flujo laminar, con precaución de no contaminar los reactivos, los cuales deben permanecer en hielo durante el tiempo de uso. Es importante evitar la exposición de la sonda a la luz directa debido a que es fotosensible y se degrada.

3. Se adicionan 21 µl de cóctel en cada vial para qPCR, seguido de 4 µl de la muestras a evaluar. Como control positivo se incluye una dilución previamente cuantificada de *R. solanacearum* y 4 µl de H_2O como control negativo, el volumen final de cada reacción es 25 µl.

4. Finalmente se llevan los tubos de PCR al equipo de Real Time PCR.

Tabla 2. Condiciones de amplificación del ADN de *R. solanacearum* mediante qPCR

Temperatura (°C)	Tiempo	No de Ciclos
95 (Denaturación inicial)	4 min	1
95 (Denaturación por ciclo)	0:30 min	
60 (Apareamiento y extensión)	1 min	40

Anexos:

1. Diseño de Sondas TaqMan® y Cebadores Mediante el Software Beacon Designer 7.5

Parámetros generales para el diseño de sondas TaqMan® y cebadores:

Longitud del amplicon: Para PCR en tiempo real es ideal tener amplicones entre 70-200bp.

Longitud de cebadores: Debe ser entre 18 y 25bp

Longitud de sonda TaqMan®: Debe ser entre 18 y 25bp

Tm °C TaqMan®: Deber estar 10 °C +/- 5 por enzima de la Tm de los cebadores.

Tm °C de cebadores: Debe estar entre 52-58 °C. Cebadores con temperaturas por encima de 65 °C tienen la tendencia a formar un annealing secundario.

Diferencias en Tm °C de los cebadores: no deben tener una diferencia de Tm superior a 3 °C.

Contenido de GC: Debe estar entre 40-60%.

Distancia entre cebador sentido y la sonda: La distancia máxima aceptable es de 10 bp.

CG Clamp: Mas de 3 bases G o C deben ser evitadas en las ultimas 5 bases del extremo 3' del cebador.

Definición de ΔG: La energía libre de Gibbs indica la cantidad de energía que se necesita para destruir la formación de una estructura secundaria. Entre mas negativo sea este valor indica que la estructura secundaria es mas estable.

Self Dimer de cebadores: Es formado por interacciones intermoleculares en donde los cebadores del mismo sentido son homólogos entre si. El ΔG tolerable para el extremo 3' es de -5 Kcal/mol e internamente un ΔG tolerable de -6 Kcal/mol.

Self Dimer TaqMan®: Es aceptable un valor máximo de -10 Kca/mol.

Cross Dimer: Es formado por interacciones intermoleculares en donde los cebadores sentido y contra sentido son homólogos entre si. El ΔG tolerable para el extremo 3' es de -5 Kcal/mol e internamente un ΔG tolerable de -6 Kcal/mol.

Hairpins de cebadores: Es formado por interacciones intramoleculares del cebador. Los valores tolerables de hairpins en el extremo 3' son de ΔG de -2 Kcal/mol y ΔG interno de -3 Kcal/mol.

Hairpins de sonda TaqMan®: Es aceptable un valor de - 6 kcal/mol.

Repeats de cebadores: Son dinucleotidos que se repiten varias veces y consecutivamente en la secuencia de los cebadores. Por ejemplo ATATATAT. Es aceptable un valor de 4 dinucleotios.

Repeats de sonda TaqMan®: Un valor de máximo 5 dinucleotidos es aceptable.

Runs de cabadores: Se denomina Runs cuando un mismo nucleótido se repite varias veces seguidas en un cebador por ejemplo: AGCGGGGGATGGGG este cebador tiene un run de 4. El máximo valor aceptable es de 4.

Runs de sonda TaqMan®: Un valor de 5 es aceptable.

Evitar la formación de estructuras secundarias en el amplicon: Cuando los cebadores se diseñan sobre una región que forma estructuras secundarias el producto y la eficiencia del PCR disminuye. Si el amplicon forma una estructura secundaria debe tener un ΔG poco negativo.

Evitar homologia cruzada: Se deben diseñar cebadores y sondas específicos evitando la homologia con secuencias afines para lograr una buena especificad. A los cebadores y a las sondas para QPCR; al igual que los amplicones se les debe revisar la homología utilizando la herramienta BLAST.

Uso del Software:

1. En la barra de herramientas busque FILE>NEW>PROJECT dar un nombre al proyecto. Por defecto, el programa da opción de guardar en la carpeta del programa pero si lo prefiere puede guardarlo en otra carpeta.

2. Luego ingrese las secuencias. Seleccione FILE>OPEN>SEQUENCE> encontrará cuatro opciones dependiendo de la ubicación de la secuencia:

- FROM ENTREZ o con click en  inmediatamente ingrese el numero de accesión. Puede ingresar varios números de accesión al mismo tiempo separados por comas.
- FROM db SNP o con click en  inmediatamente ingrese la ID, puede poner varias ID separadas por comas.
- FROM FILE o con click en  Esta opción se usa si tiene la secuencia dentro de un archivo presente en el PC o USB.
- FILE>NEW>SEQUENCE o con click en  se usa cuando se desean ingresar secuencias en forma de texto. Ingrese en SEQUENCE DEFINITION el nombre de la secuencia y luego pegue la secuencia, finalmente haga click en ADD.

3. Diseñe sondas y cebadores dependiendo de los objetivos de su investigación. El programa Beacon Desinger 7 le permite hacer sondas para discriminaciones alélicas, multiplex qPCR y genotipificación de SNPs.

El programa ofrece varias opciones de sondas: TaqMan®, Molecular Beacon, MethyLight TaqMan®, FRET probes, Scorpions®. Las opciones de sondas y cebadores son sugeridas por el programa simultáneamente.

4. Seleccione la secuencia en la tabla de información.

5. Seleccione ANALYZE>TaqMan® SEARCH>STANDARD o de click en 

6. En el cuadro de TaqMan® probe search se pueden modificar la longitud del amplicon, la Tm y la longitud de los cebadores y la sonda. Es recomendable usar los valores que tiene el programa por defecto. Si se modifica alguna opción esta es guardad para futuras búsquedas; se puede retornar a las opciones que tiene el programa por defecto escogiendo la opción DEFAULT.

7. La formación de estructuras secundarias y la homología cruzada son evitadas por el programa por defecto.

8. Inicie la búsqueda de cebadores y sonda haciendo click en Search.

9. El programa le sugerirá varias opciones de sondas y cebadores. Para verlas todas haga clic en ALL TaqMan® PROBES, observe y analice cada opción de sonda y cebadores teniendo en cuenta la longitud del amplicon, la Tm, y la formación de estructuras secundarias.

10. Si desea ver la representación grafica de las estructuras secundaria que se forman, seleccione una sonda y cebadores y haga click en ALL STRUCTURES.

11. Si desea ver la ubicación de la sonda y cebadores dentro de la secuencia haga click en REMPLACE.

12. Observe el valor de RATING, ese valor califica la sonda y cebadores escogidos teniendo en cuenta la Tm y la estabilidad de estructuras secundarias formadas por los cebadores y la sonda.

13. Una vez se escoja una sonda y cebadores que cumplan con los requerimientos de la investigación, se debe revisar la estabilidad de las estructuras secundarias formadas por el amplicon y se debe revisar la homologia e identidad mediante la herramienta BLAST.

14. Para estructuras secundarias formadas por el amplicon seleccione ANALYZE>TEMPLATE STRUCTURES SEARCH o de click en  . El rango de búsqueda de estructuras secundarias dentro de la secuencia, puede ser reducido solamente al amplicon seleccionando SEQUENCE RANGE. La temperatura de 55°C es aquella a la cual se simula la formación de estructuras secundarias.

15. Para revisar la homología e identidad seleccione ANALYZE>BLAST SEARCH, aquí va a encontrar cuatro opciones: COMPLETE SEQUENCE, PRIMER PAIR, AMPLICON Y PROBES. Es recomendable utilizar la herramienta BLAST para cada una de estas opciones.

16. Seleccione en Search type la opción nr BLAST.

17. Para generar un reporte seleccione FILE>GENERATE REPORT o de click en  ego imprima.

18. Para exportar los resultados seleccione FILE>EXPORT TaqMan® RESULTS o de click en  , luego puede seleccionar los cebadores y sondas que se van a exportar y puede seleccionar la carpeta a la cual se desean enviar los resultados.

2. Preparación de Buffers y soluciones

A. Phosphate buffered saline 10M stock solution

Sodium chloride	80g
Sodium phosphate dibasic	2g
Potassium phosphate monobasic	11.5g
Deionized water	1L

Mix all ingredients

Add 100ml of 10M stock solution to 900ml of deionized water to make 0.1M PBS.

B. PBS with 1g/L Bovine serum albumin

After autoclaving, add 1g of bovine serum albumin to sterile 0.1M PBS.

Store at 4°C.

C. Buffer fosfato 50 mM pH 7.0

Na_2HPO_4	4.26 g
KH_2PO_4	2.72 g
Deionized water	1L

Polyvinylpyrrolidone-4000 MWT (PVP-40) 5 % (Se debe adicionar después de autoclavar)

3. Equipos y reactivos

A. Bio Rad Laboratories.
IQ supermix cat # 170 8862

B. Dynal, Inc.
Magnetic particle concentrator cat # 123.01D

C. Fisher Scientific.
Bovine serum albumin fraction V cat # BP-1600-100
Potassium chloride cat # BP-366-1
Sodium phosphate dibasic cat # BP-331-1
Potassium phosphate monobasic cat # BP-362-1
Pyrex brand tubes with screw cap 13 x 100mm cat # 14-932-1A

D. Sigma-Aldrich Co.
Pectinase from *Asperillus niger* cat # P2736

E. VWR International, Inc.
Glas-col tissue culture rotator 120V cat #62404-006