Sequence Detection Systems Quantitative Assay Design and Optimization

Introduction

PE Biosystems has developed guidelines enabling streamlined design and implementation of real-time quantitative PCR assays. The use of these guidelines makes it easy to apply either the fluorogenic 5´ nuclease assay or SYBR® Green I double-stranded DNA binding dye chemistry to any real-time quantitative PCR system. Specific assay design and optimization guidelines minimize the time and cost of assay implementation, while providing reliable and robust assay performance.

Background

PE Biosystems has two instruments in its Sequence Detection Systems product line, the ABI PRISM® 7700 Sequence Detection System, and the GeneAmp® 5700 Sequence Detection System. These real-time systems are capable of detecting PCR products as they accumulate during PCR and so enable the accurate and reproducible quantitation of DNA and RNA over a wide dynamic range. With at least five orders of linear dynamic range, there is no longer a need to analyze dilutions of every sample and this, coupled with the elimination of post-PCR processing, makes real-time methodology compatible with high-throughput sample analysis.

Two chemistries are available for use on the ABI PRISM® 7700 and the GeneAmp® 5700 systems. The fluorogenic 5´ nuclease assay, or TaqMan® assay, uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR. PE Biosystems' patented fluorogenic probe design that incorporates the reporter due on the 5´ end and the quencher on the 3´ end has greatly simplified the design and synthesis of effective TaqMan probes[1,2,3]. Coupled with PE Biosystems' latest assay design guidelines, this has resulted in success rates approaching 100 percent for quantitative TaqMan® assays. The second assay chemistry available uses SYBR® Green I dye, a highly specific double-stranded DNA binding dye, which also allows the detection of product accumulation during PCR. The most important difference between the two chemistries is that SYBR® Green I assay chemistry will detect all double-stranded DNA, including non-specific reaction products. A well optimized reaction is, therefore, essential for accurate quantitative results. The advantage of SYBR® Green I assay chemistry is that no probe is required, thus reducing assay setup and running costs.

Sequence Detection Systems Assay Design and Optimization

A comprehensive set of guidelines covering assay design and optimization has been developed by PE Biosystems to ensure success when using Sequence Detection Systems instrumentation. These guidelines, however, remain simple and easy to follow. Furthermore, many variables that have required optimization in traditional PCR are kept constant, reducing assay setup and development time.

The assay design and optimization procedure contains the following important steps:

- Primer and probe design using Primer Express® software
- Selecting the appropriate reagent configuration
- Universal thermal cycling parameters
- Assay optimization

These steps provide a rapid and reliable system for assay design and optimization only when used in their entirety. The system must be adopted as a whole in order to achieve the highest level of success, due to the interdependence of many of the individual components. To illustrate this point, consider the following example. The ability to use universal thermal cycling parameters is based on the assumption that the selected primers have a melting temperature (T_m) of 58–60 °C as calculated by Primer Express® software. If the primers do not have the correct T_ms, or even if the T_ms have been calculated with a primer design software package other than Primer Express®, optimal performance and even functionality of the assay cannot be assured. Many such examples can be given, and the resulting message is always that the guidelines are best used as a complete system. Using them selectively may well lead to sub-optimal performance, and perhaps the need for extensive optimization.

Primer and Probe Design Using Primer Express® Software

Primer Express® software uses a set of default parameters to automatically select primer and probe sets. A summary of the primer and probe design guidelines is shown in Table 1.

Even though no probe is required for SYBR® Green I dye detection, it is still a good idea to use Primer Express® software to select a primer and probe set when designing a SYBR® Green I assay. Although no probe will be used, the primers will meet all the required criteria and if, in the future, there is the need to convert the assay to TaqMan® assay chemistry to obtain higher specificity, the probe can immediately be found in the original Primer Express® software document.

<table>
<tr><th>TaqMan® Probe Guidelines</th><th>Sequence Detection Primer Guidelines (SYBR® Green or TaqMan® Assays)</th></tr>
<tr><td colspan="2">Select the probe first and design the primers as close as possible to the probe without overlapping it (amplicons of 50–150 base pairs are strongly recommended)</td></tr>
<tr><td colspan="2">Keep the G/C content in the 20–80% range</td></tr>
<tr><td colspan="2">Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided</td></tr>
<tr><td>When using Primer Express® software the T_m should be 68–70 °C</td><td>When using Primer Express® software the T_m should be 58–60 °C</td></tr>
<tr><td>No G on the 5′ end</td><td rowspan="2">The five nucleotides at the 3′ end should have no more than two G and/or C bases*</td></tr>
<tr><td>Select the strand that gives the probe more C than G bases*</td></tr>
</table>

*These design parameters are not automatically screened for by Primer Express® software.

Table 1. Primer and Probes selection guidelines for quantitative assays.

An important default parameter in Primer Express® software is the selection of amplicons in the 50–150 base pair range. Small amplicons are favored because they promote high-efficiency assays that work the first time. In addition, high-efficiency assays enable relative quantitation to be performed using the comparative C_T method $(\Delta\Delta C_T)$[2]. This method increases sample throughput by eliminating the need for standard curves when looking at expression levels of a target relative to a reference control.

Whenever possible, primers and probes should be selected in a region with a G/C content of 20–80%. Regions with a G/C content in excess of this may not denature well during thermal cycling, leading to a less efficient reaction. In addition, G/C-rich sequences are susceptible to non-specific interactions that may reduce reaction efficiency and produce non-specific signal in SYBR® Green I assays. For this same reason, primer and probe sequences containing runs of four or more G bases should be avoided. A/T-rich sequences require longer primer and probe sequences in order to obtain

the recommended T_ms. This is rarely a problem for quantitative assays; however, probes approaching 40 base pairs can exhibit less efficient quenching and produce lower synthesis yields.

Selecting primers and probes with the recommended T_ms is one of the factors that allows the use of universal thermal cycling parameters. Having the probe T_m 8–10 °C higher than that of the primers ensures that the probe is fully hybridized during primer extension.

Primer Express® software does not select probes with a G on the 5′ end. The quenching effect of a G base in this position will be present even after probe cleavage. This can result in reduced normalized fluorescence values (ΔR_n), which can impact the performance of an assay. Having G bases in positions close to the 5′ end, but not on it, has not been shown to compromise assay performance. Another empirical observation is that probes with more C than G bases will often produce a higher ΔR_n. Since Primer Express® software does not automatically screen for this feature, it must be checked manually. If a probe is found to contain more G than C bases, the complement of the probe selected by Primer Express® software should be used, ensuring that a G is not present on the 5′ end.

The last five bases on the 3′ end of the primers should contain no more than two C and/or G bases, which is another factor that reduces the possibility of non-specific product formation. Under certain circumstances, however, such as a G/C-rich template sequence, this recommendation may have to be relaxed to keep the amplicon under 150 base pairs in length. It should, however, be followed as often as possible, and even when it is not possible, primer 3′ ends extremely rich in G and/or C bases should be avoided.

Selecting the Appropriate Reagent Solution

The use of the hot start enzyme AmpliTaq Gold® DNA Polymerase is an integral part of PE Biosystems' development guidelines for both TaqMan® and SYBR® Green 1 assays. The use of AmpliTaq Gold® DNA Polymerase ensures a robust reaction and can dramatically reduce the amount of non-specific product formation. A further benefit is the simplification of assay setup, which no longer needs to be performed on ice. The incorporation of AmpErase® Uracil N-glycosylase (UNG) and dUTP, which together provide protection against PCR carryover contamination, is also beneficial in ensuring a robust and high-throughput assay.

TaqMan® Assays

It is not necessary to perform titration experiments to obtain optimal concentrations of reaction components, such as magnesium chloride, when following the assay design guidelines. A generic master mix can be used for all assays when the guidelines are followed in their entirety and when reaction components are kept in excess. The additional specificity provided by the probe is a significant factor that allows TaqMan® assays to be performed in this manner. An example of a master mix formulation is TaqMan® Universal PCR Master Mix, a reagent specifically designed to provide optimal performance for TaqMan® assays that use cDNA or DNA as a substrate. This product contains components that ensure excellent assay performance even when demanding G/C-rich target sequences are encountered. The use of one reagent for all assays is another factor that simplifies the process of assay implementation.

SYBR® Green Assays

SYBR® Green I assay chemistry detects all double-stranded DNA. This means that the principle used for TaqMan® assays, in which non-specific product can be tolerated, must be modified. This is achieved by reducing the magnesium chloride concentration used in the reaction to a point where it is no longer in excess. By incorporating only this minor modification, however, a generic master mix can be used for all real-time SYBR® Green I assays.

Using the Universal Thermal Cycling Parameters

All quantitative assays designed using PE Biosystems' guidelines can be run using the same universal thermal cycling parameters. This eliminates any optimization of the thermal cycling parameters and means that multiple assays can be run on the same plate without sacrificing performance. This benefit is critical when combining two assays into a multiplex TaqMan® assay system, in which the option to run the assays under different thermal cycling parameters is not available.

Table 2 shows the universal thermal cycling parameters for quantitative TaqMan® or SYBR® Green I assays when using DNA or cDNA as the substrate.

Times and Temperatures			
Initial Steps		**Each of 40 Cycles**	
		Melt	**Anneal/Extend**
HOLD	**HOLD**	**CYCLE**	
2 min*	10 min**	15 sec	1 min
50 °C	95 °C	95 °C	60 °C

*The 2 min hold at 50 °C is required for optimal AmpErase® UNG activity

** The 10 min hold at 95 °C is required for AmpliTaq Gold® DNA Polymerase activation

Table 2. Universal Thermal Cycling Parameters for real-time quantitative TaqMan® or SYBR® Green I Assays.

Optimizing Primer Concentrations

By independently varying forward and reverse primer concentrations, the concentrations that provide optimal assay performance can be identified. Primers are always in large molar excess during the exponential phase of PCR amplification, and by adjusting their initial concentration their effective T_ms can be adjusted. By spanning an initial concentration range of 50 nM–900 nM for each primer, the effective T_m of the primers can be adjusted by approximately plus or minus 2 °C from the midpoint, see Table 3. This enables the primer optimization matrix to compensate for non-specific primer binding, which can reduce the amount of primer available to bind at its specific site. In the same way, the matrix is able to compensate for small errors in T_m estimation by Primer Express® software . The primer concentrations used in the primer optimization matrix are shown in Table 3.

Reverse Primer (nM)	**Forward Primer (nM)**		
	50 *(-2 °C)*	**300** *(0 °C)*	**900** *(+2 °C)*
50 *(-2 °C)*	50/50	300/50	900/50
300 (*0 °C)*	50/300	300/300	900/300
900 *(+2 °C)*	50/900	300/900	900/900

Table 3. Primer Optimization Matrix.

TaqMan® Assays

For a TaqMan® assay, optimal performance is achieved by selecting the primer concentrations that provide the lowest C_T and highest ΔR_n for a fixed amount of target template. The results of a typical TaqMan® primer optimization matrix experiment are shown in Figure 1. Figure 1(a) shows the amplification plots for all nine primer concentration combinations in linear view, and Figure 1(b) shows the same data in log view format. The combination of 50 nM forward and reverse primer (Plot Group C) gives both the lowest ΔR_n and highest C_T. All other primer combinations that contain a 50 nM concentration of either the forward or reverse primer (Plot Group B) give a reduced ΔR_n. All primer combinations that contain at least 300 nM forward and reverse primer (Plot Group A) give both the highest ΔR_n and the lowest C_T; as a result, any of these combinations would provide optimal performance. It should be noted that, although C_T values are the parameter by which quantitation values are assigned in a real-time quantitative PCR assay, ΔR_n values can also prove important when trying to obtain maximum sensitivity and reproducibility.

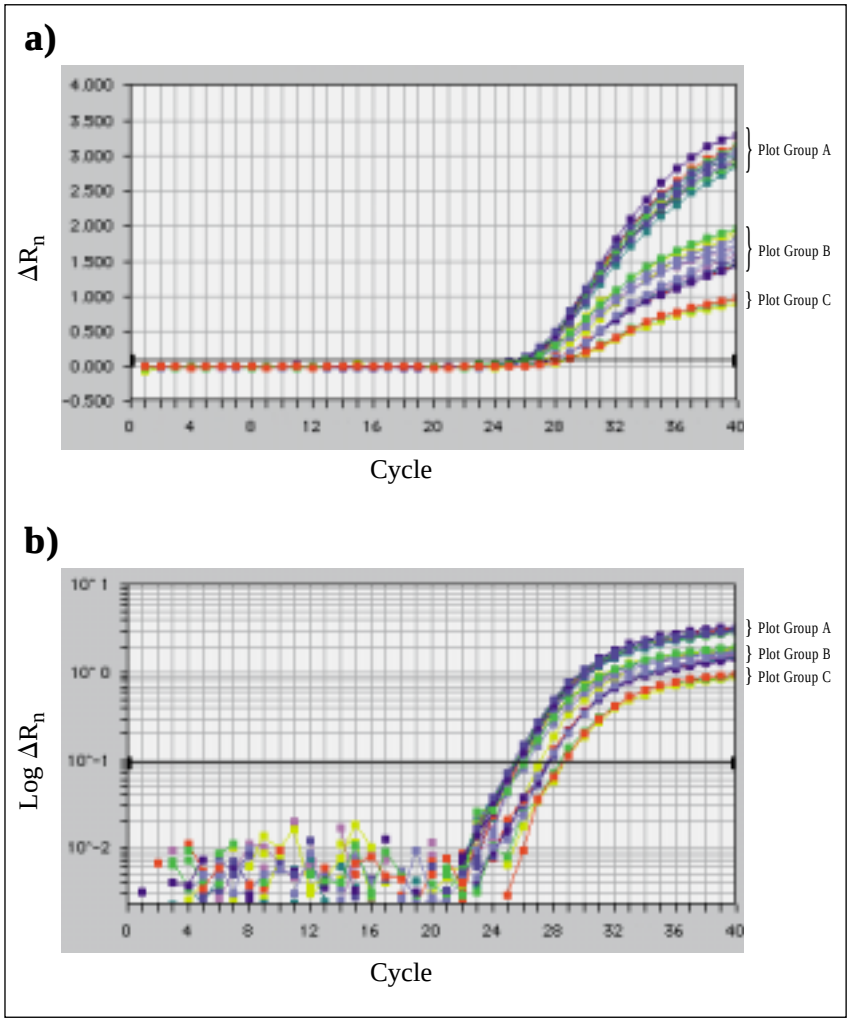

Figure 1. Primer optimization experimental results showing amplification plots of all nine primer combinations in the Primer Optimization Matrix. (a) linear view (b) log view.

Plot Group A: combinations that contain at least a 300 nM concentration of forward and reverse primer.

Plot Group B: combinations that contain a 50 nM concentration of forward or reverse primer.

Plot Group C: 50 nM concentrations of forward and reverse primer.

SYBR® Green I Assays

Optimizing primer concentrations is slightly more complex for a SYBR® Green I assay. The same primer optimization matrix should be performed; however, this time it should not only be run with a fixed amount of target template, but the same matrix should also be challenged with NTCs (no template controls). In this case, the primer concentrations selected should provide a low C_T and high ΔR_n when run against the target template, but should also minimize non-specific product formation when challenged with NTCs . Dissociation curves or gel analysis can be extremely useful when selecting optimal primer concentrations for a SYBR® Green I assay. This is demonstrated in Figure 2, which shows the results from a primer optimization matrix at primer concentrations of 900 nM forward and reverse primers. The strong amplification of the NTC wells shown in Figure 2(a) indicates that significant non-specific amplification is occurring. This is confirmed by the dissociation curve data shown in Figure 2(b), which shows that the melting temperature of the product generated in the absence of template is lower than the melting temperature of the specific product generated with template. This is typical of primer-dimer formation and indicates that lower primer concentrations should provide more optimal results with a larger linear dynamic range.

Probe Concentration Optimization for TaqMan® Assays

Using the optimal primer concentrations defined by the primer optimization matrix ensures excellent assay performance when using a 250 nM probe concentration. However, depending on the requirements of the assay, a probe optimization experiment can prove useful. Figure 3 shows the results of a probe optimization experiment in which the probe concentration is varied from 50–250 nM. Figure 3(a) shows an increase in ΔR_n as the probe concentration is increased, whereas Figure 3(b) shows that the C_T value is unchanged for all concentrations of probe except 50 nM. This allows for the possibility that the assay can be run at a probe concentration under 250 nM (but above 50 nM), with a resulting reduction in assay running costs. It should be noted, however, that to ensure the best reproducibility, especially when wishing to detect low copy numbers of a target sequence, it is necessary to avoid probe limiting concentrations. By using a 250 nM concentration, probe limitation is avoided and large ΔR_n values are ensured.

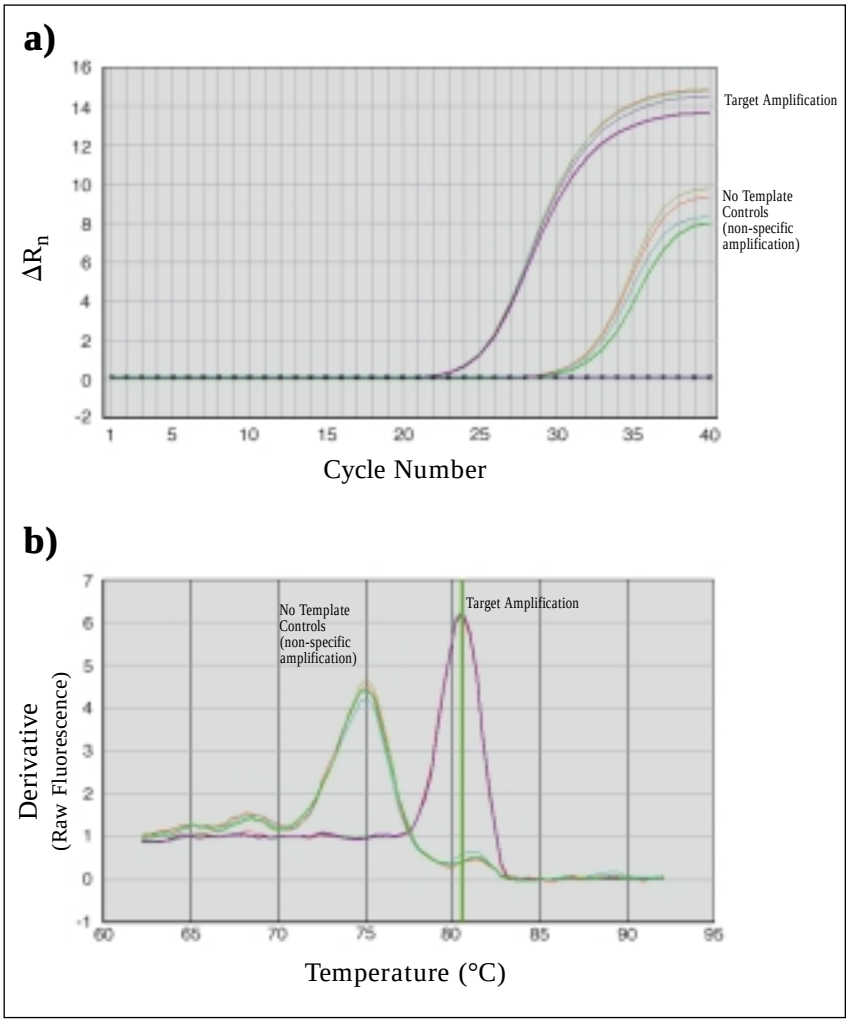

Figure 2. Amplification data using SYBR® Green I assay chemistry. (a) amplification plot (linear view) demonstrating suspected non-specific amplification in NTC (no template control) wells. (b) Dissociation curve analysis confirming product in NTC wells has a different melting temperature from specific product. Dissociation curve analysis is performed after a completed PCR. Data is obtained by slowly ramping the temperature of reaction solutions from 60 to 95 °C while continuously collecting fluorescence data. The increase in temperature causes PCR products to undergo denaturation, a process accompanied by a decrease in fluorescence for solutions containing SYBR® Green I dye. By plotting the first derivative of fluorescence versus temperature, the melting profile of distinct PCR products can be obtained, as shown in Figure 3(a).

Primer Limitation for Multiplex TaqMan® Assays

Multiplex TaqMan® assays can be performed on the ABI PRISM® 7700 Sequence Detection System[3] due to its capability to detect multiple dyes with distinct emission wavelengths. The most common application in which this is used is relative quantitation of gene expression, in which one probe labeled with FAM dye is used to detect the target species and another probe, labeled with VIC™ dye, is used to detect an endogenous control gene. Running both assays in a single tube reduces both the running costs and the dependence on accurate pipetting when splitting a sample into two separate tubes. To generate an accurate multiplex assay, it is important to ensure that the amplification of one species does not dominate the other. If this is not done, the amplification of a highly abundant species can prevent the less abundant species from amplifying efficiently. Such a scenario could easily produce inaccurate results and, in severe cases, may inhibit detection of the less abundant species completely. This situation can be avoided by limiting the concentrations of the primers used to amplify the more abundant species, so "turning off" the amplification soon after the C_T has been established. Primer limitation results in the reaction components common to both assays not being exhausted, allowing the amplification of the less abundant species to continue at high efficiency. If the more abundant species is not known,

it should be determined before entering into a multiplex assay system by running both targets in separate tubes. Both amplifications should be primer limited if neither species is consistently most abundant.

To determine primer limiting concentrations, the limiting primer matrix shown in Table 4 should be performed. The goal is to identify primer concentrations that reduce the ΔR_n of the assay without affecting the C_T value.

The results of a limiting primer matrix experiment are shown in Figure 4. Figure 4(a) shows that only when lowering the primer concentrations below approximately 50 nM is the C_T value significantly affected. Figure 4(b) shows the corresponding relationship between primer concentrations and ΔR_n, and demonstrates that lower product yields can be achieved by decreasing forward and reverse primer concentrations. The plateau area visible in Figure 4(a) shows the region in which suitable primer limiting concentrations can be found. In this area the C_T, and therefore the corresponding quantitation value, are unchanged, whereas the ΔR_n value and corresponding product yield are significantly reduced. For this example, an appropriate selection of primer limiting concentrations would be 60 nM forward and reverse primer. It is important to note that probe concentration should be kept at an optimal level even when an assay is primer limited to ensure that the signal produced is large enough for accurate multicomponenting by the ABI PRISM® 7700 Sequence Detection System software.

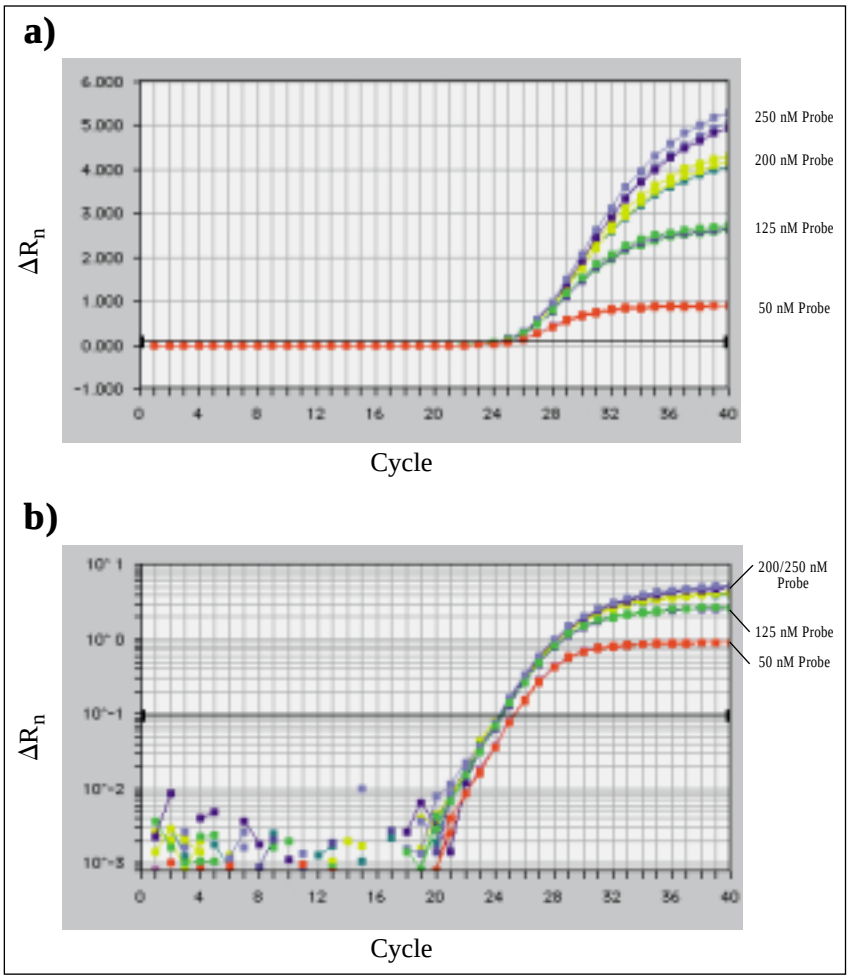

Figure 3. Amplification plot (linear and log views) of probe concentration titration from 50–250 nM.

Reverse Primer Concentration (nM)	**Forward Primer Concentration (nM)**				
	20	**40**	**60**	**80**	**100**
20	20/20	40/20	60/20	80/20	100/20
40	20/40	40/40	60/40	80/40	100/40
60	20/60	40/60	60/60	80/60	100/60
80	20/80	40/80	60/80	80/80	100/80
100	20/100	40/100	60/100	80/100	100/100

Table 4. Limiting Primer Matrix: Varying concentrations of forward and reverse primers (20–100 nM).

Although following all design criteria does facilitate the ability to identify limiting primer concentrations, it may not be possible for all assays. If a limiting primer matrix experiment does not enable the identification of primer limiting concentrations, it will be necessary to redesign at least one primer or run the reactions in separate tubes.

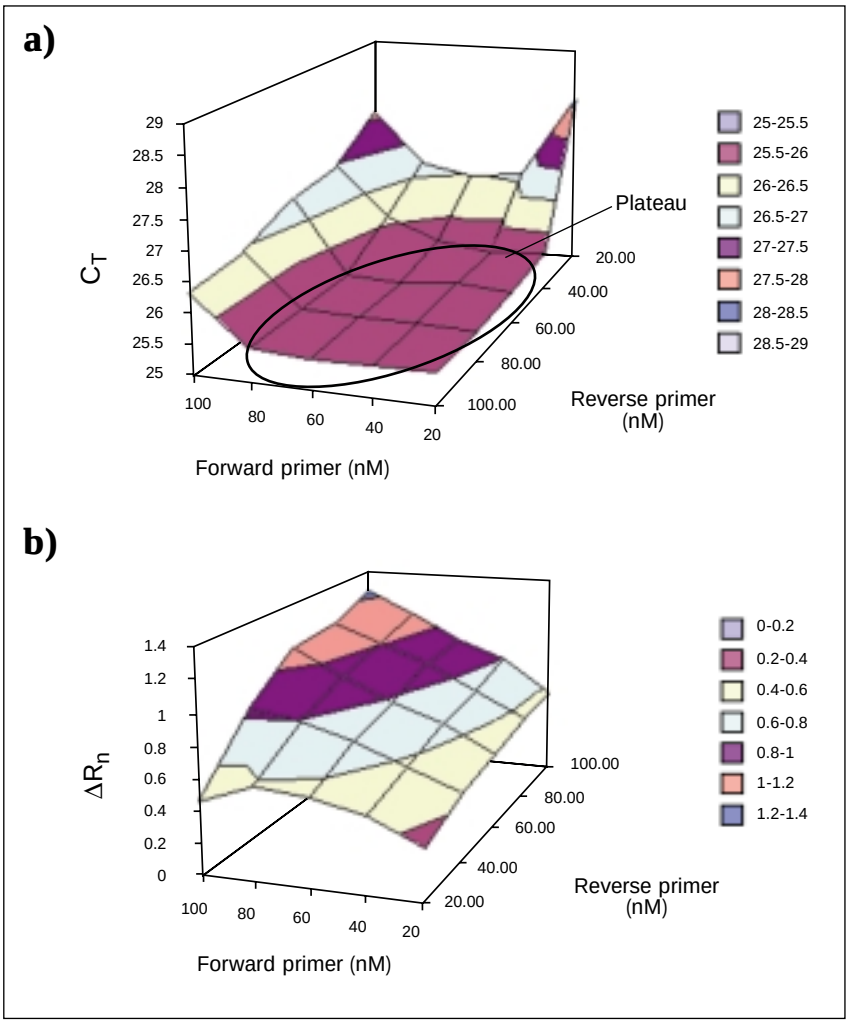

Figure 4. Results from Limiting Primer Matrix experiment. (a) shows how C_T value is affected by variation in forward and reverse primer concentrations. Plateau region indicated shows area where C_T value remains constant. (b) shows reduction in ΔR_n values as primer concentrations are decreased.

Summary

The PE Biosystems assay development guidelines enable quantitative assays to be designed and optimized rapidly and efficiently. Since thousands of assays have been developed this way, the following conclusion can be made. For the vast majority of quantitative TaqMan® assays designed and run following PE Biosystems assay development guidelines, using a concentration of 900-nM primers and 250-nM probe will provide for a highly reproducible and sensitive assay when using cDNA or DNA as a substrate. Due to the non-specific nature of its detection, SYBR® Green I primer optimization should be eliminated only with caution. However, if all guidelines are followed, concentrations of 50 nM forward and reverse primer should provide robust amplification with a good level of specificity when using DNA or cDNA as a substrate. This assumption should, however, always be verified by checking for non-specific product formation with either dissociation curve or gel analysis.

PE Biosystems' assay development guidelines do not guarantee that all assays will provide the same level of performance and sensitivity. Even the most scrupulous design parameters can not account for all the possible variables that can exist between two different assay systems. As a general rule, however, a real-time quantitative TaqMan® assay should enable detection and accurate quantitation down to at least 500 copies of a target sequence, with much greater sensitivity possible. A real-time SYBR® Green I assay is capable of similar performance; however, non-specific product formation can potentially increase the minimum detection limit.

References

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