Identification of Nonspecific Products Using Melt-Curve Analysis on the iCycler iQ™ Detection System

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Introduction

Real-time PCR* is a powerful and effective technique for accurate quantitation of DNA. Assays for the detection of a single gene involve careful choice of primers, target sequence, and the method for detecting the amplified DNA product. In addition, it is necessary to choose appropriate reaction conditions that will generate robust and efficient amplification for both relative and absolute quantitation.

Specificity and efficiency are two essential requirements for successful real-time PCR amplification. Poor assay design can result in such problems as nonspecific products and inefficient primer association with target, which dramatically affect the quality of the data. Proper primer design ensures that the chosen primers are specific for the desired target sequence, bind at positions that avoid secondary structure, and minimize the occurrence of primer-dimer formation. Appropriate reaction conditions can also improve the efficiency of amplification. Optimization of the detection method, Mg^{2+} concentration, annealing temperature, enzyme concentration, PCR product length, etc., are all ways of perfecting real-time assays.

Another valuable tool in assay design is melt-curve analysis. With the use of DNA-binding dyes such as SYBR Green I, a melt-curve profile can be generated. The iCycler iQ system records the total fluorescence generated by SYBR Green I binding to double-stranded DNA as temperature changes, and plots the fluorescence in real time as a function of temperature. The first derivative of this plot, dF/dT, is the rate of change of fluorescence in the reaction, and a significant change in fluorescence accompanies the melting of the double-stranded PCR products. A plot of -dF/dT vs. temperature will display these changes in fluorescence as distinct peaks. The melting temperature (T_m) of each product is defined as the temperature at which the corresponding peak maximum occurs. This analysis can confirm the specificity of the chosen primers as well as reveal the presence of primer-dimers. Because of their small size, primer-dimers usually melt at lower temperatures than the desired product. Additionally, nonspecific amplification may result in PCR products that melt at temperatures above or below that of the desired product.

The presence of secondary nonspecific products and primer-dimers can severely reduce the amplification efficiency and ultimately the accuracy of the data. Primer-dimers can also limit the dynamic range of the desired standard curve due to competition for reaction components during amplification. Therefore, melt-curve analysis is essential in designing an efficient and specific quantitative PCR assay.

Methods

Standard Curves with IL-1β

The following master mix was prepared to generate standard curves with the IL-1β plasmid template (IMAGE Consortium clone 324655) using SYBR Green I as the detection reagent:

1 ml Platinum PCR Super mix (Life Technologies)
5 µl 100 µM forward primer (see Table)
5 µl 100 µM reverse primer (see Table)
200 µl SYBR Green I (1:10,000 dilution of Molecular Probes stock)
750 µl ddH_2O

Alternatively, the same components as above were used except that 4 µl of a dual-labeled probe (see Table) and 196 µl of ddH_2O were used in place of SYBR Green I to detect the amplified DNA.

A series of consecutive 5-fold dilutions of the IL-1β plasmid in ddH_2O was made, creating five dilutions from 10^4 to 16 copies of the plasmid. The master mix was dispensed in 294 µl aliqouts to six separate tubes. Then a 6 µl aliquot from each plasmid dilution was added to one of the tubes containing master mix, with the last tube receiving 6 µl of ddH_2O as a no-template control.

Primers for the experiment in Figure 7 were redesigned to prevent primer-dimer formation. The following master mix was used to generate this standard curve using SYBR Green I as the detection agent:

1.425 ml Platinum PCR Super mix (Life Technologies)
8.55 µl 100 µM forward primer (see Table)
8.55 µl 100 µM reverse primer (see Table)
285 µl SYBR Green I (1:10,000 dilution of Molecular Probes stock)
1.065 ml ddH_2O

Table. Primers and probes designed to test the effects of primer dimerization on IL-1β standard curves.*

Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Fluorescent Detection Agent	Associated Figures
GTGCTGAATGTGGACTCAATCCC	GGTTGCTCATCAGAATGTGGG	SYBR Green I	1–5
GTGCTGAATGTGGACTCAATCCC	GGTTGCTCATCAGAATGTGGG	Texas Red-CAGGCCTCTCTCA-CCTCTCCGCCTGG-DABCYL	6
TGCTCCTTCCAGGACCT	GTGGTGGTCGGAGATTC	SYBR Green I	7
CTTTGCCGAGTCAGGAACA	GACTTCTGCACGCTCC	6-FAM-CGCTGCACTTCGAGA-TTTCCAAGGAAGGCAG-BHQ-1	8–9
CTTTGCCGAGTCAGGAACA	GACTTCTGCACGCTCC	SYBR Green I	8–9

* Primers used for experiments in Figures 1–6 were designed to induce primer-dimers by a GGG/CCC overlap at the 3' ends.

A series of consecutive 10-fold dilutions of the IL-1β plasmid template DNA ranging from 10^7 copies/µl to 10^2 copies/µl was generated. Master mix was dispensed in 343 µl aliquots to seven separate tubes. Each of the tubes then received a 7 µl aliquot of one of the DNA dilutions. ddH_2O (7 µl) was added to the last tube as a no-template control.

Aliquots (50 µl) of the reaction mixtures were transferred to the wells of a 96-well thin-wall PCR plate. The plate was sealed with optically clear sealing film and spun briefly. Each plate was subjected to a PCR amplification protocol of 95°C denaturation for 3 min followed by 40–45 cycles of 10 sec at 95°C and 45 sec at 55–60°C (annealing and data collection step) in Bio-Rad's iCycler iQ system. The protocol for Figures 4 and 5 had an additional step where data were collected at 82°C rather than at the 60°C annealing step. Melt-curve analysis was performed immediately after the amplification protocol under the following conditions: 1 min denaturation at 95°C, 1 min annealing at 55°C, 80 cycles of 0.5°C increments (10 sec each) beginning at 55°C (data collection step).

Standard Curves with β-Inhibin

The following master mix was used to produce standard curves from genomic DNA using β-inhibin primers:

2.125 ml Platinum PCR Super mix (Life Technologies)
12.75 µl 100 µM forward primer (see Table)
12.75 µl 100 µM reverse primer (see Table)
1.419 µl ddH_2O

Aliquots of 1.596 ml of master mix were dispensed to two separate tubes. To the first tube, 3.8 µl of a dual-labeled probe (6-FAM/BHQ-1, see Table) plus 186.2 µl of ddH_2O were added to detect amplified DNA. To the second tube, 190 µl of a 1:10,000 dilution of SYBR Green I were added for DNA detection (final dilution 1:100,000).

Genomic DNA was diluted from a 66.7 ng/µl stock to generate a 4-fold dilution series from 50 to 3.125 ng/µl. The genomic DNA stock was previously digested with *Bam*HI and boiled for 10 min before being placed in an ice bath. Aliquots of 423 µl from either the 6-FAM/BHQ-1 or SYBR Green I master mix were dispensed to four separate tubes. Aliquots (27 µl) of each genomic DNA dilution or ddH_2O were then added to one tube from the 6-FAM/BHQ-1 mix and one tube from the SYBR Green I mix.

Aliquots (50 µl) of the reaction mixtures were transferred to two 96-well thin-wall PCR plates, one for each detection strategy. The plates were sealed with optically clear sealing film and spun briefly. Both plates were subjected to similar amplification and melt-curve protocols differing only in their annealing temperatures at the data collection step of amplification. The amplification steps were 95°C denaturation for 3 min, 50 cycles of 10 sec at 95°C, 45 sec at 57°C or 55°C (data collection step); melt-curve steps were 95°C denaturation for 1 min, 55°C annealing for 1 min, 80 cycles of 0.5°C increments (10 sec each) beginning at 55°C (data collection step).

Results

Primer-Dimers

A standard curve (5-fold dilution series) of IL-1β plasmid was generated with SYBR Green I using primers known to produce primer-dimers (GGG/CCC overlap at the 3' ends; see Table). The amplification data revealed that the last three dilutions could not be resolved from one another (Figure 1). In addition, amplification was also present in the no-template controls. The standard curve demonstrated an amplification efficiency above 100% (slope = -2.449 or 156% efficiency) and a poor correlation coefficient (r = 0.950). An amplification efficiency of >90% is typically desired for optimal results. Greater than 100% efficiency suggests that more than one product was amplified in the reaction. Melt-curve analysis was performed in conjunction with the amplification protocol to determine if nonspecific products were amplified during the reaction. The melt-curve results revealed a large product peak in the no-template control wells at a melting temperature (T_m) of approximately 78°C (Figure 2A). This peak was generated completely by amplified primer-dimer products. At the highest plasmid concentration (10^4 copies/well), only one major peak was apparent at a T_m of 89°C (Figure 2B). This peak represented the specific amplified product. With the lower dilutions of amplified IL-1β DNA plasmid (for example, 4 x 10^2 copies/well; Figure 2C) the melt curve revealed the presence of both the primer-dimer and the specific product, or the primer-dimer only. The melt-curve data were confirmed by resolving the amplified products on a polyacrylamide gel (Figure 3). This analysis demonstrates that primer-dimers are present in all of the amplification reactions. Since SYBR Green I nonspecifically detects all double-stranded DNA, it will not distinguish between the specific amplified product and the primer-dimers formed during the reaction. Therefore,

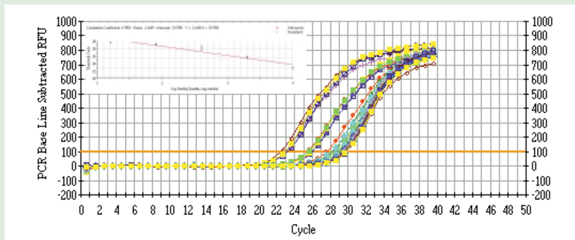

Fig. 1. Amplification of a dilution series of IL-1β plasmid with SYBR Green I detection, using a primer set designed to generate primer-dimers. The inset represents the standard curve of five plasmid dilutions.

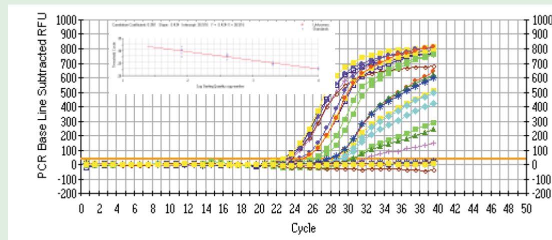

Fig. 4. Amplification with SYBR Green I and the same IL-1β primers with an annealing temperature of 60°C and data collection set at 82°C. The inset represents the standard curve of five plasmid dilutions.

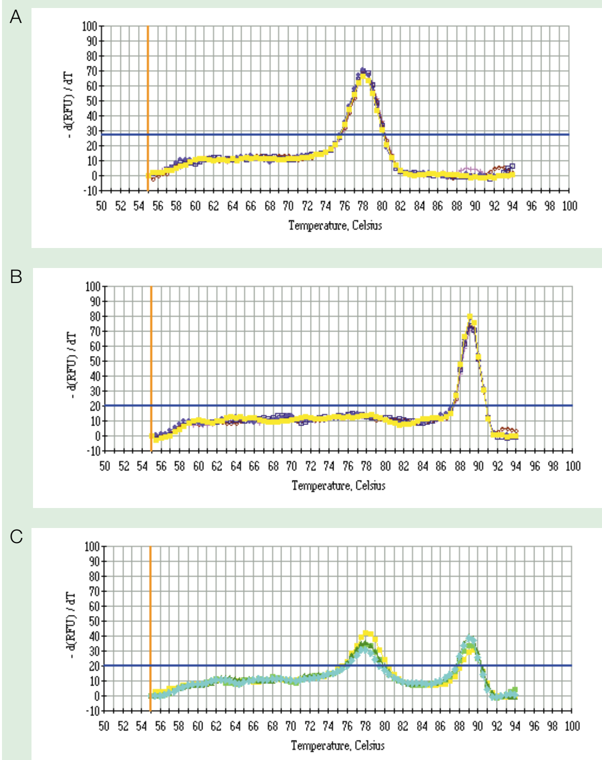

Fig. 2. Melt-curve plots representing products from the amplification shown in Figure 1. No-template control (A), 10^4 copies of IL-1β template (B), and 4×10^2 copies of IL-1β template (C).

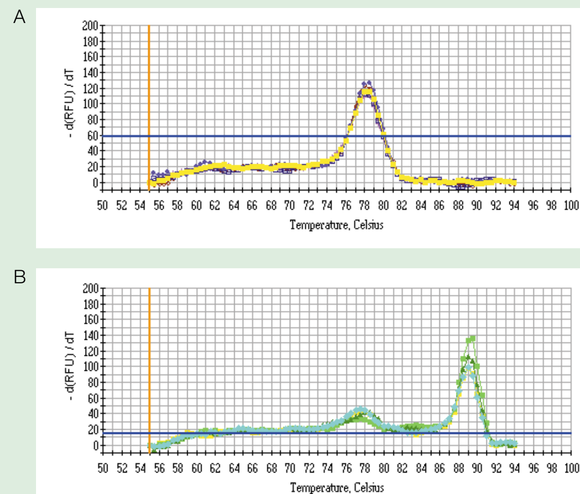

Fig. 5. Melt-curve profiles representing products generated from the amplification seen in Figure 4. No-template control (A), 2×10^4 copies of IL-1β template (B).

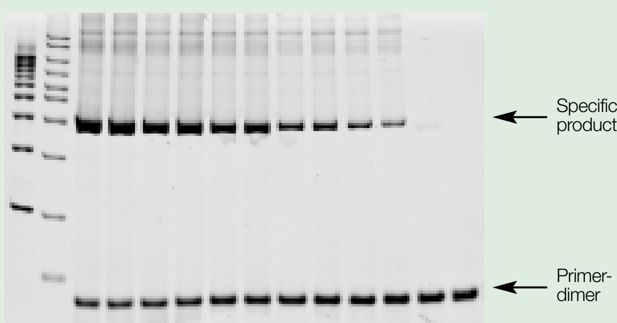

Fig. 3. Ready Gel® 15% acrylamide TBE gel showing products from the amplifications of Figure 1. Lane 1, EZ Load™ 100 bp ladder; lane 2, AmpliSize® 50–2,000 bp molecular ruler; lanes 3 and 4, 1×10^4 copies IL-1β template/reaction; lanes 5 and 6, 2×10^3 copies IL-1β ; lanes 7 and 8, 4×10^2 copies IL-1β template/reaction; lanes 9 and 10, 80 copies IL-1β template/reaction; lanes 11 and 12, 16 copies IL-1β template/reaction; lanes 13 and 14, no-template controls.

the efficiency will be >100%. The amount of the primer-dimers produced is also dependent on the original concentration of the target DNA template. When no template is present, a large and significant amount of the primer-dimer product is present. At the lower plasmid concentrations, the predominant product is still the primer-dimer. However, at higher concentrations, the specific amplified product predominates. Therefore, primer-dimer production will interfere with accurate quantitation of the target DNA by SYBR Green I. Furthermore, both the primer-dimer and the specific product are in direct competition for the reaction components during amplification. This indicates that primer-dimer formation will directly inhibit amplification of a specific product resulting in a diminished dynamic range (Figure 1).

A frequently suggested solution to primer-dimer interference is to collect amplification data at a temperature where the primer-dimers have melted, but the specific products have not. Then, the primer-dimers would not contribute to the fluorescence recorded during amplification. For the experiment depicted in Figure 4, the same steps were repeated (annealing temperature remained at 60°C); however, the data were collected in an additional step at 82°C after the primer-dimers had melted. Fluorescence was

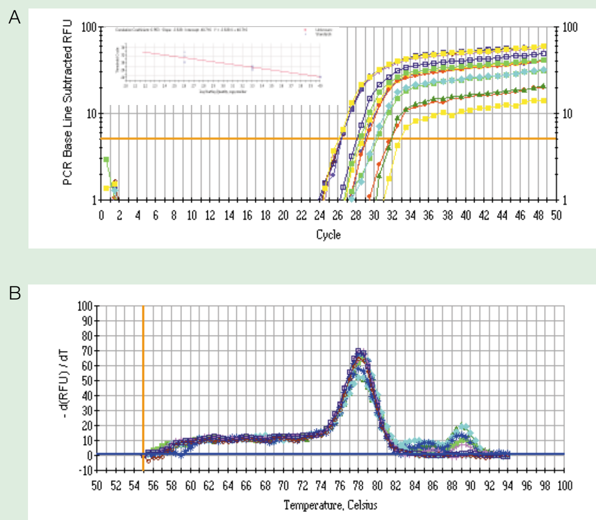

Fig. 6. Primer-dimer inhibition of specific product formation. A, dilution series of IL-1β plasmid amplification with a dual-labeled hybridization probe using the same primer set; the standard curve is shown in the inset. B, melt curves with SYBR Green I representing all primer-dimer products from amplifications with 16 or 80 copies of IL-1β template.

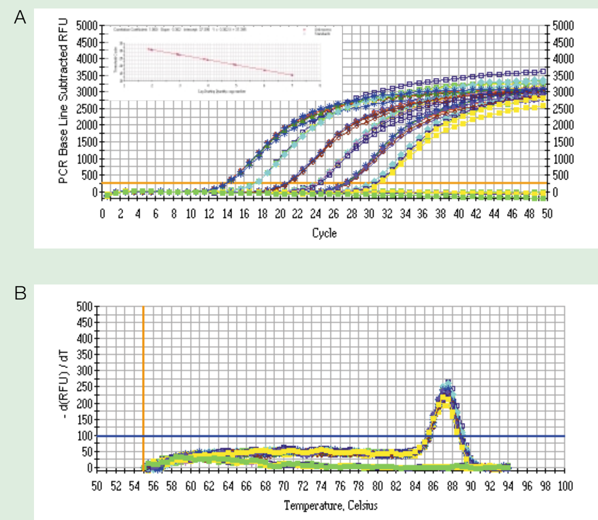

Fig. 7. Amplification (A) and melt-curve (B) profiles resulting from a 10-fold standard curve amplification with a redesigned primer set for IL-1β. The inset in (A) represents the standard curve generated over 5 orders of dynamic range.

not detected in the no-template control during amplification. This is to be expected since the primer-dimers were denatured at this temperature and could no longer contribute to the fluorescence. The lower plasmid dilutions could also be resolved from one another. Since SYBR Green I does not detect primer-dimers at 82°C, a normal amplification efficiency was revealed (slope = -3.424 or 95.9% efficiency). However, the correlation coefficient remained poor (r = 0.951). Collecting data at 82°C removes the fluorescent contribution of the primer-dimers in the amplification plot. However, it does not remove the primer-dimers themselves during amplification. As seen in Figures 5A and 5B, the primer-dimer peak is still present in the no-template control and in the various plasmid dilutions during amplification. The primer-dimers still compete for reaction components and inhibit amplification of the specific product. This is further demonstrated when the same amplification is performed using a dual-labeled probe (Texas Red/DABCYL) (Figure 6A). This probe should detect only the specific IL-1β amplified product and should not recognize the primer-dimers. The experiment resulted in an extremely diminished standard curve with a very low efficiency (r = 0.953, slope = -3.638 or 88.3% efficiency). The lowest dilutions (80 and 16 copies) could not be included in the calculations for the standard curve because the amplification of the specific product was severely or completely inhibited by the primer-dimers (Figure 6B). Collecting data after the melting temperature of the primer-dimers does not correct for their interference during amplification. Therefore, experiments in the presence of significant primer-dimers will result in inefficient and inaccurate data.

There are several possible solutions to prevent primer-dimer formation. The experiments above used primers that were known to produce primer-dimer products based on a GGG/CCC overlap at the 3' ends. Primer-dimers can be avoided by proper design. Using hot-start enzymes also greatly reduces primer-dimer formation. Furthermore, the annealing temperature as well as the reaction components themselves (eg., Mg^{2+}, detergents, SYBR Green I concentration) can also affect the amount of primer-dimers produced during amplification. If a set of primers is consistently causing problems, redesigning the primers themselves may be the best choice. Figure 7 shows a standard curve over a 10-fold difference in concentration of the same IL-1β plasmid after redesigning the primers. This amplification demonstrated an excellent correlation coefficient (r = 1.00) with a very high efficiency (slope = -3.362, 98.2% efficiency). After melt-curve analysis, it is apparent that one major product was amplified during the reaction that represents the specific product (Figure 7B). In this case, a simple redesign of the primers themselves greatly improved the quality and accuracy of the data obtained during amplification.

Secondary Products

A 4-fold dilution series of human genomic DNA was subjected to amplification with SYBR Green I using primers for the β-inhibin gene. In the amplification plot, the three dilutions in the series were resolved fairly well from one another (Figure 8A). The standard curve revealed a good correlation coefficient (r = 0.994) but a relatively poor slope value (-2.515, 150% efficiency). After melt-curve analysis, it was apparent that the >100% efficiency was caused by amplification of more than one product (Figure 8B). Two major peaks appeared in all three plasmid dilutions. The presence of nonspecific products was further confirmed by running the amplification products

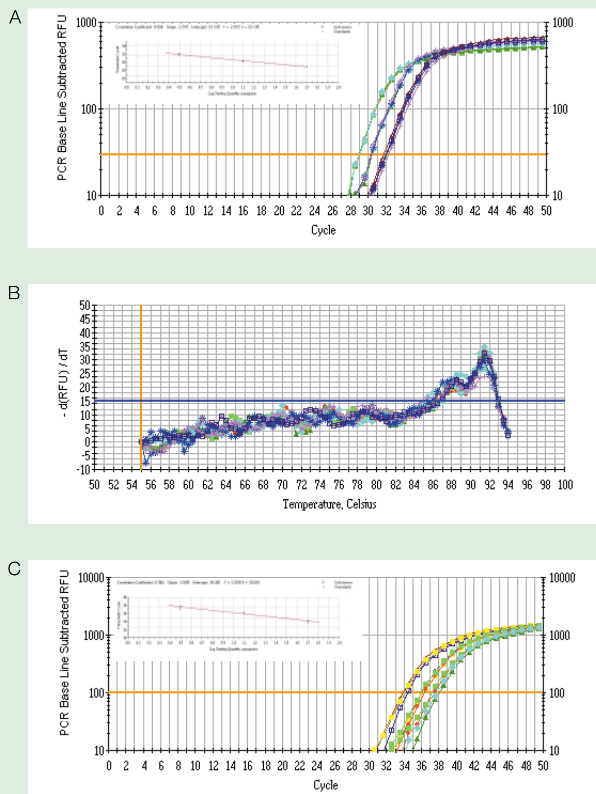

Fig. 8. Amplification of human genomic DNA at 57°C using SYBR Green I (A) or a dual-labeled hybridization probe (C) with primers recognizing the β-inhibin gene. Insets represent the standard curves generated from three plasmid dilutions. The melt-curve diagram (B) was generated from the amplification shown in panel A.

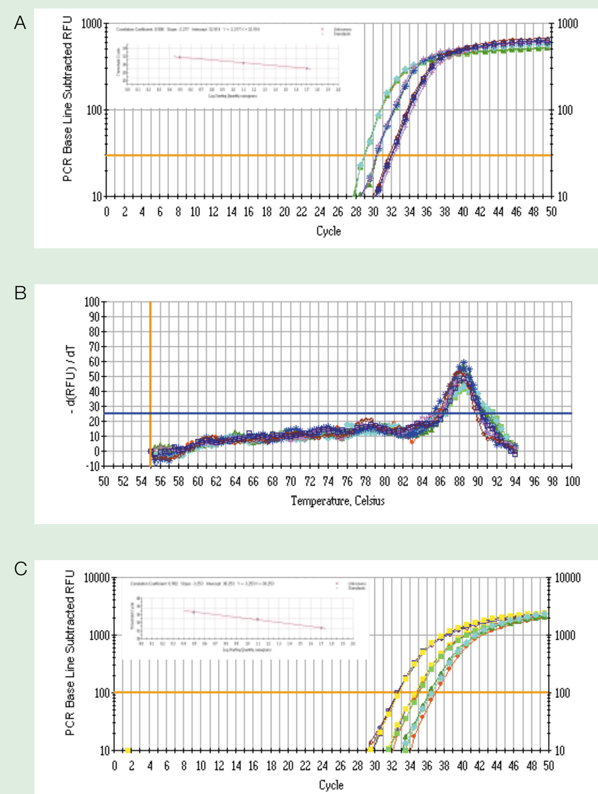

Fig. 9. Amplification of human genomic DNA at 55°C using SYBR Green I (A) or a dual-labeled hybridization probe (C) with primers recognizing the β-inhibin gene. Insets represent the standard curves generated from three plasmid dilutions. The melt-curve diagram (B) was generated from the amplification shown in panel A.

on a 15% acrylamide TBE gel. The gel revealed two major bands: a 200 bp band corresponding to the specific amplified product and a 350 bp secondary product (data not shown). The presence of the nonspecific product decreases the efficiency of amplification of the specific product due to competition for reagents. However, the ratio between the secondary product and specific product in this experiment remained the same regardless of the DNA dilution. For this reason, the secondary product did not have a greater impact on the lower concentrations of DNA as in the case of primer-dimers (see Figures 1–2).

The same amplification was performed using a dual-labeled probe (6-FAM/BHQ-1) to detect amplification of the specific product only. Figure 8C demonstrates a trend similar to that seen with SYBR Green I. Since the probe should detect only the specific 200 bp product, the standard curve would be expected to be superior to that seen with nonspecific detection by SYBR Green I. However, the correlation coefficient was poor (r = 0.982) and the efficiency of the amplification remained over 100% (slope = -2.890, 121.8%). This indicates that the coamplification of the nonspecific product influenced the amplification efficiency of the specific product, possibly because the probe could not distinguish between the specific and nonspecific products in the reaction (r = 0.982).

A simple method for reducing secondary product formation is to try different annealing temperatures. It is often found that optimizing the annealing temperature for amplification will increase primer specificity and eliminate secondary products. In this example, the annealing temperature was lowered from 57°C to 55°C. It is evident from the amplification data (Figure 9A) that reducing the annealing temperature greatly improved the correlation coefficient of the standard curve (r = 0.996). Based on the melt-curve data, it seems this improvement is caused by the elimination of the major nonspecific product (Figure 9B). Only one peak appeared in the melt curve, indicating that only one product was amplified. However, the amplification efficiency remained over 100% (slope = -2.277, 175.4%). The amplified products were separated on a 15% acrylamide TBE gel, which confirmed one major product at 200 bp (data not shown). However, several smaller secondary products were also present that could not be resolved by the melt curve. The melting of each product in the melt curve is based on both the length and the G/C content of the sequence. Because of this, not all amplified products can be resolved from one another. Therefore, a single peak in a melt curve does not necessarily mean a single product. In this example, the melt peak was quite broad (Figure 9B). This indicates that some, if not all, of the minor products may have approximately the same melting temperature as the 200 bp specific product.

When the dual-labeled probe was used in place of SYBR Green I for detection, the correlation coefficient (r = 0.992) remained similar (Figure 9C). However, the slope (-3.253, 102% efficiency) and hence the efficiency was greatly improved. The smaller secondary products at the 55°C annealing temperature had a greater impact on the SYBR Green I experiments since this detection dye is nonspecific. The probe, however, was able to distinguish between the specific product and the secondary products, leading to improved results. Therefore, it is important to minimize amplification of nonspecific products as well as to choose the appropriate detection agent.

Conclusions

In real-time PCR assays, it is important to amplify the target gene at high efficiency. This is important for generating the best possible dynamic range for standard curves and more importantly for multiplexing with other genes. Melt-curve analysis assists in improving real-time assays because it identifies the products amplified in a particular reaction. Nonspecific amplification and primer-dimer formation can greatly reduce amplification efficiency of the target gene since they compete for reaction components during amplification. The greatest effect is observed at the lowest concentrations of DNA, which ultimately compromises the dynamic range. Since primer-dimers directly interfere with target gene amplification, collecting data at a higher temperature after primer-dimers have melted could lead to inaccurate data. Optimization of reaction components (Mg^{2+}, detergents, SYBR Green I concentrations) and annealing temperatures will aid in decreasing nonspecific product formation. However, proper primer design is the best method to avoid primer-dimers and other nonspecific products (refer to bulletin 2593, General Considerations for PCR).

* The polymerase chain reaction (PCR) process is covered by patents owned by Hoffman-LaRoche. Use of the PCR process requires a license. The iCycler iQ system includes a licensed thermal cycler and may be used with PCR licenses available from Applera. Its use with authorized reagents also provides a limited PCR license in accordance with the label rights accompanying such reagents. Some applications may require licenses from other parties.

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