DNA/RNA Real-Time Quantitative PCR

The polymerase chain reaction (PCR) has revolutionized the detection of DNA and RNA. As little as a single copy of a particular sequence can be specifically amplified and detected. Theoretically, there is a quantitative relationship between amount of starting target sequence and amount of PCR product at any given cycle. In practice, though, it is a common experience for replicate reactions to yield different amounts of PCR product. The development of real-time quantitative PCR has eliminated the variability traditionally associated with quantitative PCR, thus allowing the routine and reliable quantitation of PCR products.

History of Real-Time PCR Techniques

Higuchi *et al.*[1,2] pioneered the analysis of PCR kinetics by constructing a system that detects PCR products as they accumulate. This "real-time" system includes the intercalator ethidium bromide in each amplification reaction, an adapted thermal cycler to irradiate the samples with ultraviolet light, and detection of the resulting fluorescence with a computer-controlled cooled CCD camera. Amplification produces increasing amounts of double-stranded DNA, which binds ethidium bromide, resulting in an increase in fluorescence. By plotting the increase in fluorescence versus cycle number, the system produces amplification plots that provide a more complete picture of the PCR process than assaying product accumulation after a fixed number of cycles.

Chemistry Developments For Real-Time PCR

Fluorogenic Probes

Real-time systems for PCR were improved by probe-based, rather than intercalator-based, PCR product detection. The principal drawback to intercalator-based detection of PCR product accumulation is that both specific and nonspecific products generate signal. An alternative method, the 5' nuclease assay,[3,4] provides a real-time method for detecting only specific amplification products. Holland *et al.*[3] were the first to demonstrate that cleavage of a target probe during PCR by the 5' nuclease activity of *Taq* DNA polymerase could be used to detect amplification of the target-specific product. In addition to the components of a typical amplification, reactions included a probe labeled with ^{32}P on its 5' end and blocked at its 3' end so it could not act as a primer. During amplification, annealing of the probe to its target sequence generates a substrate that is cleaved by the 5' nuclease activity of *Taq* DNA polymerase when the enzyme extends from an upstream primer into the region of the probe. This dependence on polymerization ensures that cleavage of the probe occurs only if the target sequence is being amplified. After PCR, Holland *et al.* measured cleavage of the probe by using thin layer chromatography to separate cleavage fragments from intact probe.

The development of fluorogenic probes by Lee *et al.*[5] made it possible to eliminate post-PCR processing for the analysis of probe degradation. The probe is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by Förster resonance energy transfer (FRET) through space. Probe design and synthesis has been simplified by the finding that adequate quenching is observed for probes with the reporter at the 5' end and the quencher at the 3' end.[6]

Figure 1 diagrams what happens to a fluorogenic probe during the extension phase of PCR. If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of *Taq* DNA polymerase as this primer is extended. This cleavage of the probe separates the reporter dye from quencher dye, increasing the reporter dye signal. Cleavage removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process. Additional reporter dye molecules are cleaved from their respective probes with each cycle, effecting an increase in fluorescence intensity proportional to the amount of amplicon produced.

The advantage of fluorogenic probes over DNA binding dyes is that specific hybridization between probe and target is required to generate fluorescent signal. Thus, with fluorogenic probes, non-specific amplification due to mis-priming or primer-dimer artifact does not generate signal. Another advantage of fluorogenic probes is that

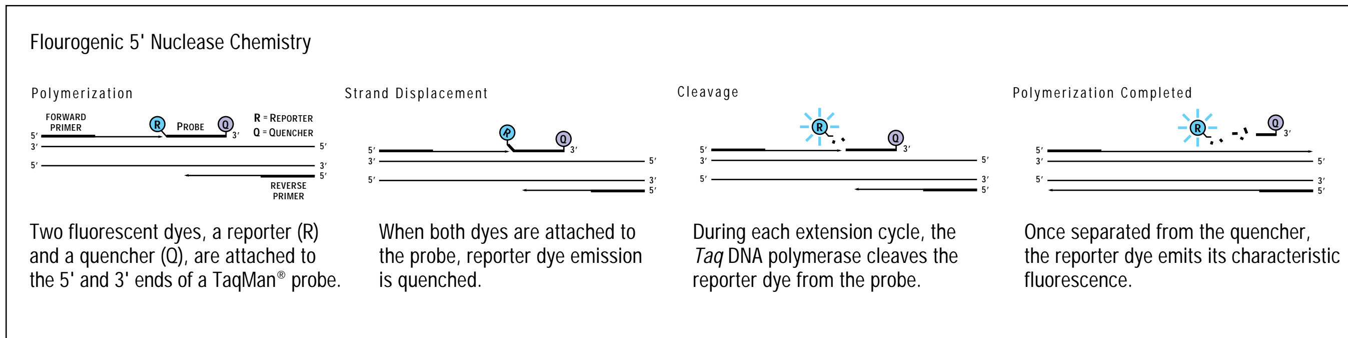

Figure 1. *Stepwise representation of the forklike-structure–dependent, polymerization-associated, 5' to 3' nuclease activity of* Taq *DNA polymerase acting on a fluorogenic probe during one extension phase of PCR.*[7]

they can be labeled with different, distinguishable reporter dyes. By using probes labeled with different reporters, amplification of two distinct sequences can be detected in a single PCR reaction. The disadvantage of fluorogenic probes is that different probes must be synthesized to detect different sequences.

Double-Stranded DNA Binding Dyes

Small molecules that bind to double-stranded DNA can be divided into two classes: intercalators and minor groove binders.[8] Higuchi *et al.* used the intercalator ethidium bromide for their real-time detection of PCR. Hoeschst 33258 is an example of a minor groove binding dye whose fluorescence increases when bound to double-stranded DNA.[9] Regardless of binding mechanism, there are two requirements for a DNA binding dye for real-time detection of PCR: 1) increased fluorescence when bound to double-stranded DNA; 2) no inhibition of PCR. PE Biosystems has developed conditions that permit the use of the SYBR® Green I dye[10] in PCR without inhibition and with increased sensitivity compared to ethidium bromide. The mechanism of SYBR® Green I dye's interaction with DNA (intercalator vs. groove binding) is not known.

Both the advantage and disadvantage of using a DNA binding dye for real-time detection of PCR are that the dye allows detection of any double-stranded DNA generated during PCR. On the plus side, this means versatility because the same dye can be used to detect any amplified product. Thus, any PCR amplification can be monitored simply by including the generic DNA binding dye with the other PCR reagents. On the negative side, both specific and non-specific products generate signal. Thus, any mis-priming events that lead to spurious bands observed on electrophoretic gels will generate false positive signal when a generic DNA binding dye is used for real-time detection.

Another aspect of using DNA binding dyes is that multiple dyes bind to a single amplified molecule. This increases the sensitivity for detecting amplification products. A consequence of multiple dye binding is that the amount of signal is dependent on the mass of double-stranded DNA produced in the reaction. Thus, if the amplification efficiencies are the same, amplification of a longer product will generate more signal than a shorter one. This is in contrast to the use of a fluorogenic probe, in which a single fluorophore is released from quenching for each amplified molecule synthesized, regardless of its length.

Instrumentation

PE Biosystems has two instruments designed to detect fluorescence during the thermal cycling of PCR. The simpler system is the GeneAmp® 5700 Sequence Detection System. This complete system consists of an Optical Detector and a GeneAmp® PCR System 9600, coordinately controlled by software running on a Windows®-based computer. The system has been designed for efficient detection of PCR product accumulation using either SYBR® Green I double stranded DNA binding dye or TaqMan® fluorogenic probes. The 96 reaction tubes are irradiated with a white light source and the resulting fluorescence is detected using a CCD array to capture an image of all 96 wells. The software collects the images throughout the thermal cycling of PCR and analyzes the data to generate an amplification plot for each reaction. Fluorogenic probes labeled with fluoroscein can be detected on the 5700 system, but the instrument does not have the capability of distinguishing two or more fluorophores. Despite this single-color detection limitation, however, the 5700 system is still able to use an internal reference dye (ROX) to

normalize for non-PCR-related, well-to-well fluctuations in fluorescence. This ability to normalize is achieved by using fluorescence readings taken at 95 °C in the baseline region and is essential for reproducible results.

The ABI PRISM® 7700 Sequence Detection System is a more flexible system designed to take full advantage of the benefits of fluorogenic probe detection. The 7700 system has a built-in thermal cycler and a laser directed via fiber optic cables to each of the 96 sample wells. The fluorescence emission travels back through the cables to a CCD camera detector. Because each well is irradiated sequentially, the dimensions of the CCD array can be used for spectral resolution of the fluorescent light. This contrasts with the 5700 system, in which the CCD is used for spatial resolution of the 96 wells. Because the 7700 instrument detects an entire fluorescence spectrum, the system is capable of distinguishing and quantitating multiple fluorophores in each sample well. The software analyzes the data by first calculating the contribution of each component dye to the experimental spectrum. Each reporter signal is then divided by the fluorescence of an internal reference dye (ROX) in order to normalize for non-PCR related fluorescence fluctuations occurring well-to-well or over time. The use of this internal reference dye, enabled by the ability to distinguish fluorophores, increases the precision of the data obtained with the 7700 system. The fluorescence emissions of SYBR® Green I dye and ROX dye are well resolved, so the benefit of using an internal reference dye is obtained for SYBR® Green I dye detection of PCR on the 7700 system. The other advantage of distinguishing fluorophores is that probes labeled with different reporter dyes can be used so that more than one PCR target can be detected in a single tube.

Real-Time PCR Quantitation

The ability to monitor the real-time progress of the PCR completely revolutionizes the way one approaches PCR-based quantitation of DNA and RNA. Reactions are characterized by the point in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

Figure 2 shows a representative amplification plot and defines the terms used in the quantitation analysis. An amplification plot is the plot of fluorescence signal versus cycle number. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter C_T (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. As shown by Higuchi *et al.*[2], a plot of the log of initial target copy number for a set of standards versus C_T is a straight line. Quantitation of the amount of target in unknown samples is accomplished by measuring C_T and using the standard curve to determine starting copy number. The entire process of calculating C_Ts, preparing a standard curve, and determining starting copy number for unknowns is performed by the software of the 5700 and 7700 systems.

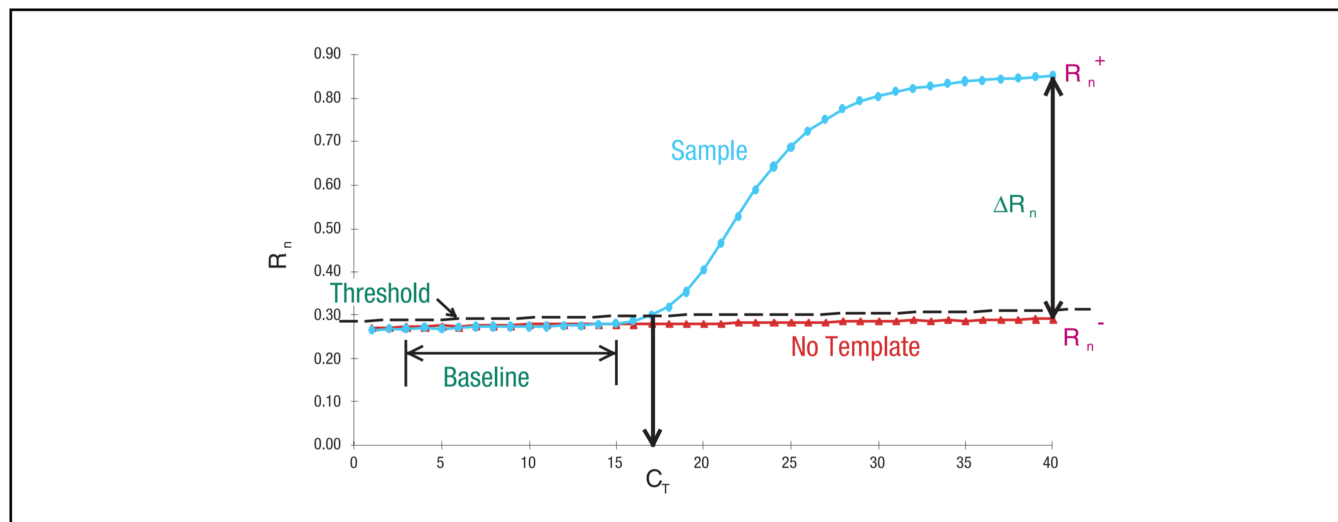

Figure 2. *Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR.*

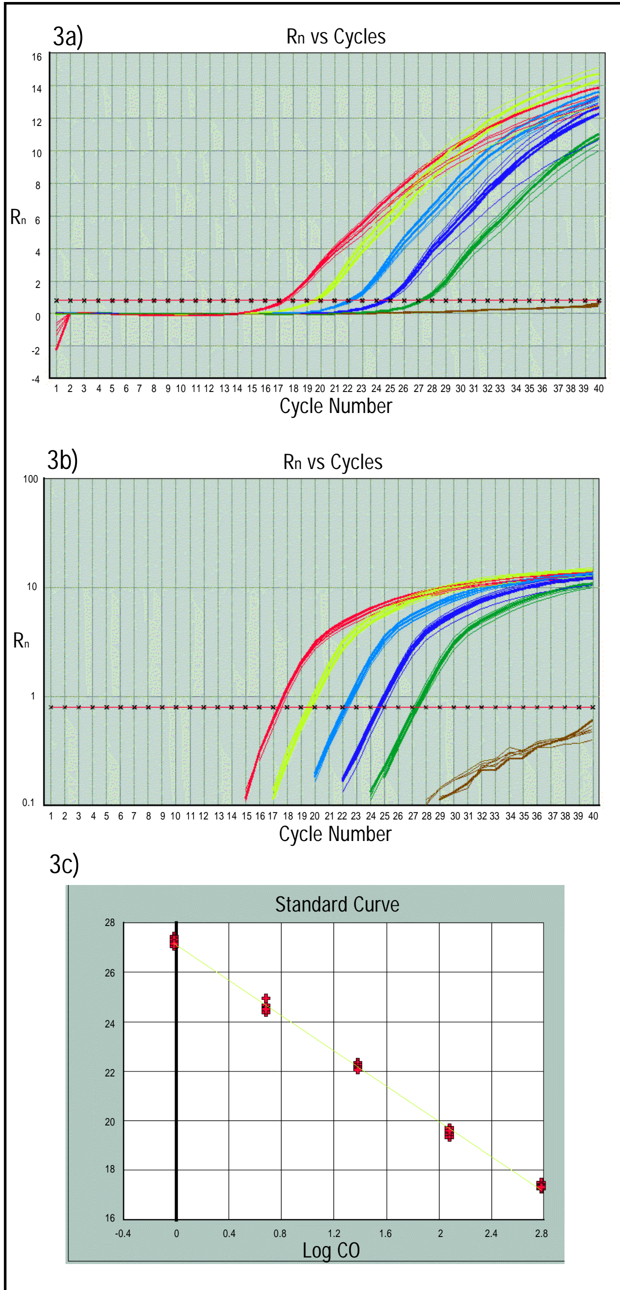

Figure 3. *Amplification of the human β-action gene in five-fold dilutions of genomic DNA using the GeneAmp® 5700 Sequence Detection System. (a) Amplification plot showing the change in fluorescence of SYBR® Green I dye plotted versus cycle number. (b) Same data showing the log of the change in fluorescence plotted versus cycle number. (c) Standard curve showing C_T values plotted versus the log of the initial amount of genomic DNA.*

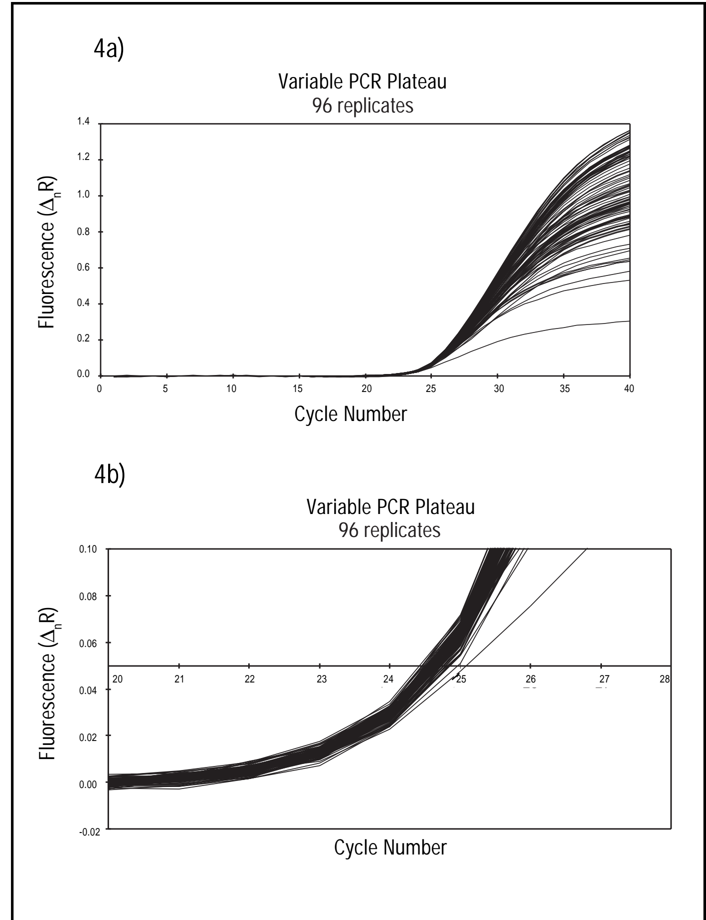

Figure 4. *Amplification of a segment of the β-actin gene from human genomic DNA. Samples contained 10 ng human genomic DNA (corresponds to 3300 copies of a single copy gene) and were amplified using the components of TaqMan PCR Reagent Kit (PE Biosystems). (a) Amplification plots of 96 replicates. (b) Detail of cycles 20–28. The abscissa is placed at a ΔR_n value of 0.05 to show the threshold used for calculation of C_T. The average final ΔR_n value at cycle 40 is 1.03 ± 0.22 (c.v. = 21.4%). The average C_T value is 24.64 ± 0.11. A standard deviation of 0.11 for C_T corresponds to a c.v. of 7.9% for calculated starting copy number. Experiment performed by Traci Allen, PE Biosystems.*

Figure 3a shows amplification, using the 5700 system, of the human β-actin gene in five-fold dilutions of genomic DNA. In this figure, the change in fluorescence of SYBR® Green I dye is plotted versus cycle number. Six replicates were run for each DNA amount. Figure 3b shows the same data, but with the log of the change in fluorescence plotted versus cycle number. The 5700 system software calculated the C_T (threshold cycle) for each reaction. The C_T values are plotted versus the log of the initial amount of genomic DNA to give the standard curve shown in Figure 3c.

These three plots illustrate the basic principles of real-time PCR quantitation. The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the C_T value. C_T values are very reproducible in replicates because the threshold is picked to be in the exponential phase of the PCR. This is shown in Figure 3b where the threshold intersects the amplification plots in the region where there is a linear relation between log of the change in fluorescence and cycle number. In the exponential phase, reaction components are not limiting and replicate reactions exhibit uniform and reproducible results.

Effect of Limiting Reagents

The early cycles of PCR are characterized by an exponential increase in target amplification. As reaction components become limiting, the rate of target amplification decreases until a plateau is reached and there is little or no net increase in PCR product. The sensitive fluorescence detection of the 5700 and 7700 systems allows the threshold cycle to be observed when PCR amplification is still in the exponential phase. This is the main reason why C_T is a more reliable measure of starting copy number than an endpoint measurement of the amount of accumulated PCR product. During the exponential phase, none of the reaction components is limiting; as a result, C_T values are very reproducible for reactions with the same starting copy number. This leads to greatly improved precision in the quantitation of DNA and RNA. On the other hand, the amount of PCR product observed at the end of the reaction is very sensitive to slight variations in reaction components. This is because endpoint measurements are generally made when the reaction is beyond the exponential phase and a slight difference in a limiting component can have a drastic effect on the final amount of product. For example, side reactions, like formation of primer dimers, can consume reagents to different extents from tube to tube. Thus, it is possible for a sample with a higher starting copy number to end up with less accumulated product than a sample with a lower starting copy number. The differences between endpoint and real-time detection are graphically illustrated in Figure 4, which shows amplification of 96 identical samples. The overall change in reporter signal, as measured at cycle 40, varies widely among the replicates. However, the amplification plots are remarkably similar between cycles 22 and 25, during which the C_T values are determined.

Quantitative Competitive PCR

In order to compensate for problems with endpoint measurements, researchers have developed a variety of quantitative competitive PCR techniques.[11–14] Typically, a competitor amplicon is constructed that contains the same primer binding sites and has the same amplification efficiency as the target, but is somehow distinguishable from the target. A common distinguishing characteristic is to make the target and competitor amplicons different sizes so that gel electrophoresis can be used to discriminate the two products. A known amount of competitor is spiked into the sample, then the target and competitor are amplified in the same reaction. If the amplification efficiency of target and competitor are, in fact, identical, then the ratio of target to competitor will remain constant throughout the PCR process. Thus, by determining the ratio of target to competitor at the end of the reaction and knowing the starting amount of competitor spiked in, the starting amount of target can be calculated. Competitive PCR has been used successfully to quantitate DNA and RNA, but its dynamic range is limited to a target-to-competitor ratio of about 1:10 to 10:1. In fact, the best accuracy is obtained by finding the equivalence point at which the ratio of target to competitor is 1:1. To accomplish this, several dilutions must be tested in order to achieve a suitable ratio of target to competitor. Another drawback is the need to construct and characterize a different competitor for every target to be quantitated. In addition, careful validation studies must be performed in order to verify that the amplification efficiencies of target and competitor are the same before quantitation of experimental samples can commence. Even a slight difference in efficiency severely compromises the accuracy of quantitation by competitive PCR. At the end of the reaction, competitive PCR requires accurate quantitation of target and competitor amplicons, which usually entails laborious post-PCR processing steps.

Advantages of Real-Time

The development of competitive PCR was driven by a reliance on endpoint measurements. Determining C_T values by following the real-time kinetics of PCR eliminates the need for a competitor to be co-amplified with the target. Quantitation can be performed by the more basic method of preparing a standard curve and determining unknown amount by comparison to the standard curve. Compared to endpoint measurements, the use of C_T values also expands the dynamic range of quantitation because data is collected for every cycle of PCR. A linear relationship

between C_T and initial DNA amount has been demonstrated for over five orders of magnitude, compared to the one or two orders of magnitude typically observed with an endpoint assay. Real-time quantitation eliminates post-PCR processing of PCR products, which not only increases throughput and reduces the chances for carryover contamination, but also removes post-PCR processing as a potential source of error. Although not immune, C_T values are less sensitive than endpoint values to the effects of PCR inhibitors, again, because measurements are from the exponential phase where reaction components are not limiting.

Quantitation of Cytokine Gene Expression

One application of the real-time quantitative capability of either the GeneAmp® 5700 Sequence Detection System or the ABI PRISM® 7700 Sequence Detection System is to monitor how patterns of gene expression change in response to various stimuli. Figure 5 shows the relative quantitation results for three cytokine mRNAs in PBMCs that have been stimulated with CD3/CD28 antibodies. Total RNA was prepared from untreated PBMCs and from cells that had been exposed to CD3/CD28 antibodies for 2 hours, 6 hours, or overnight. The relative quantity of IL-2, IL-4, and TNF-α mRNA in each sample was determined using either generic SYBR® Green I dye detection or gene-specific fluorogenic probes. Thermal cycling and detection of the real-time amplification plots were performed using the 5700 and the 7700 Sequence Detection Systems. In order to perform these analyses, cDNAs prepared from the four total RNA samples were mixed with either TaqMan® Universal PCR Master Mix or a master mix formulated from the components of the SYBR® Green PCR Core Reagents Kit. These master mixes contain all necessary reaction components except primers and probes. Each cDNA mix was added to separate wells, each well containing the primer pair for amplification of one of the cytokine targets. Samples containing the primer pair for amplification of 18S ribosomal RNA were also prepared. For gene-specific detection, the wells also contained the appropriate fluorogenic probe for the cytokine or ribosomal RNA target.

Analysis of multiple targets on the same plate requires that all primer sets work using the same thermal cycling parameters. The primer pairs for the cytokine targets were selected using the PE Biosystems Guidelines for 'Amplifying Custom Target Sequences for Quantitation'.[15] Following these guidelines allows the amplification of all targets to be performed using the same reaction conditions and thermal cycling parameters. Adhering to these guidelines also means that the efficiency of each PCR amplification is close to 1. Because the efficiencies of the different amplifications are approximately equal and close to 1, it is possible to obtain relative quantitative results without having to run standard curves for any of the cytokine targets. Detailed procedures for relative quantitation are described in ABI PRISM® 7700 Sequence Detection System User Bulletin No. 2.[16] Figure 5 shows the relative quantitation results comparing the four samples for the three cytokine targets. In this analysis, the untreated sample was designated the calibrator and it is assigned the value of 1× for each of the targets. The quantity of each cytokine mRNA in each of the treated samples is given relative to the calibrator sample.

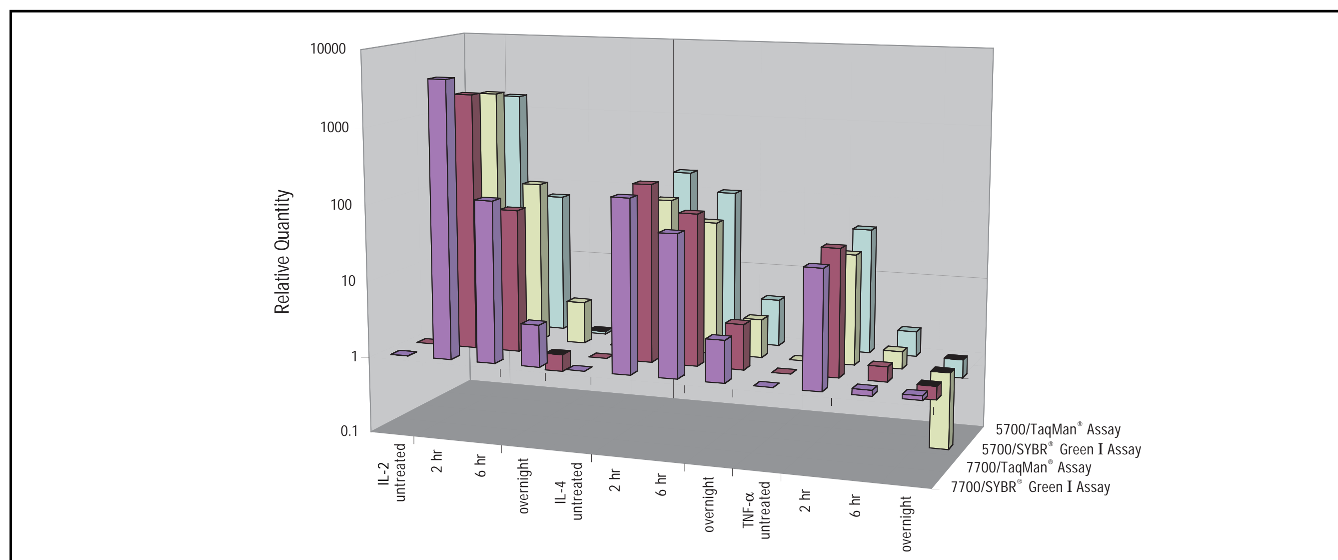

Figure 5. *Relative quantitation results for three cytokine mRNAs in PBMCs that have been stimulated with CD3/CD28 antibodies. (Data generated in collaboration with DNAX Research Institute.)*

The results shown in Figure 5 were normalized using the C_T values obtained for the ribosomal RNA amplifications run in the same plate. The 18S ribosomal RNA is an endogenous control that is used to normalize the samples for differences in the amount of total RNA added to each cDNA reaction. The use of this endogenous reference also normalizes for variation in reverse transcriptase (RT) efficiency among the different cDNA reactions. Variation in RT efficiency other than sample-to-sample variation is controlled for because a single cDNA reaction is performed for each total RNA sample. This single cDNA reaction is then split to perform the target and control amplifications.

At high levels of RNA, Figure 5 shows that approximately the same relative quantitation results are obtained whether SYBR® Green I dye detection or fluorogenic probe detection is used on either the 5700 or 7700 system. Thus, at the 2 hr time point, the increase in cytokine mRNA level is approximately 2000-fold for IL-2, 100-fold for IL-4, and 40-fold for TNF-α, regardless of analysis method used. At low levels of RNA, though, the detection of non-specific amplification by SYBR® Green I dye complicates the results. For the IL-2 analysis in the overnight sample, fluorogenic probe detection on either the 5700 or 7700 system shows the level of IL-2 mRNA is about 0.75× the amount in the untreated sample. Using SYBR® Green I dye detection, the amount of IL-2 mRNA in the overnight sample appears to be 3.5×. This inflated value observed with SYBR® Green I dye detection is due to the detection of non-specific amplification products. The TNF-α levels observed in the overnight sample reveal a different consequence of non-specific amplification. Both analyses using fluorogenic probe detection indicate the TNF-α level is about 0.6× compared to the untreated sample. The two SYBR® Green I dye results give wildly different values of 1× and 0.03×. Non-specific amplification is not necessarily consistent well-to-well, so replicates can give much different results. Thus, non-specific amplification can lead to erroneous and/or highly variable results at low target levels when SYBR® Green I dye or another generic DNA-binding dye is used for detection. For these particular cases, lower primer concentrations were tried to increase the specificity of the amplifications. Changing primer concentrations from 900 nM to 50 nM reduced the spurious results observed with SYBR® Green I dye detection.

The use of fluorogenic probes avoids the complications caused by detection of non-specific amplification. Because non-specific amplification is more of a problem at low target levels, fluorogenic probe assays tend to be more sensitive for detection of low amounts of target and have a greater dynamic range compared to assays using DNA binding dyes. Another advantage of fluorogenic probes is that, on the ABI PRISM® 7700 system, the target and endogenous control (e.g., rRNA) amplification can be performed in the same tube. This is possible because target and control probes can be labeled with distinguishable reporter dyes. This reduces the number of reactions that need to be run and ensures that exactly the same amount of template is available for target and control amplification. Also, the inclusion of an in-tube internal positive control increases confidence in the results obtained for target quantitation.

Detection on the ABI PRISM® 7700 system tends to give lower coefficients of variation than detection on the GeneAmp® 5700 system. Improved precision means that smaller differences in initial copy number can be distinguished.

Conclusions

Compared to endpoint quantitation methods, real-time PCR offers streamlined assay development, reproducible results, and a large dynamic range. Real-time PCR eliminates the need for competitive in-tube standards with identical primer sets as targets. Thus, the process of creating quantitative assays is streamlined because the construction and characterization of such standards are no longer required. Real-time PCR now makes quantitation of DNA and RNA much more precise and reproducible because it relies on C_T values determined during the exponential phase of PCR rather than endpoint. In addition, the use of C_T values allows a larger dynamic range. This increases throughput because it is no longer necessary to analyze dilutions of each sample in order to obtain accurate results.

The researcher now has a number of options for implementing real-time quantitation in his or her lab. Homogeneous detection of PCR products can be done using double-stranded DNA binding dyes or fluorogenic probes. Detection of fluorescence during the thermal cycling process can be performed using either the GeneAmp® 5700 or ABI PRISM® 7700 Sequence Detection Systems. Choosing among these options requires balancing the demands of sensitivity, convenience, precision, and cost.

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