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EDITORIAL

Real-Time Quantitative PCR

Those who have worked in the field of quantitative polymerase chain reaction (PCR) since the early 1990s have accepted many of the tedious aspects of the early assays as routine. Difficulties associated with early quantitative PCR techniques included: (*i*) ensuring that the PCR was within the linear range of amplification (that portion where the PCR signal is directly proportional to the input copy number) and (*ii*) finding a suitable method to detect the product once linear amplification was achieved. To ensure linearity, researchers would amplify serial dilutions of cDNA (1) or alter the amplification cycle for each gene (2). Others would add a competitor template (3), perform limiting dilution assays (4), or use a PCR mimic with a similar primer sequence that would be coamplified along with the product (5). Detection of the amplicon generally resorted to running gels and DNA detection using a stain, radioactivity, or probe.

For those who have converted to using real-time quantitative PCR, the old days are but a memory. In real-time PCR, worrying about linear amplification, running gels, working with radioactivity, and gathering numbers are a thing of the past. The ability to generate data within a 2- to 3-h period is now a reality. Other advantages of real-time PCR include enhanced sensitivity, high throughput, use of a closed-tube system, reduced variation, the ability to simultaneously multiplex reactions, and lack of post-PCR manipulations.

The technology to detect PCR products in real time, i.e., during the reaction, has been available for more than 5 years, but has seen a dramatic increase in use over the past 2 years. A Medline search using the key words *TaqMan* or *real-time* and *PCR* yielded 19 citations in 1996, 28 citations in 1997, and 52, 157, and 409 citations in 1998, 1999, and 2000, respectively. At the time of this writing, there were 551 citations in 2001 (Fig. 1). Various manufacturers are touting real-time PCR instrumentation and affiliated technology (softwear, probes, etc.) and more are likely to be released in the near future.

In this thematic issue, Real-Time Quantitative PCR, we bring together many of the techniques that are currently being used to quantify DNA or RNA using real-time PCR. We have assembled nine articles from leaders in the field ranging from quantification of mRNA and viral DNA to the amplification and detection of DNA methylation patterns and small nucleotide polymorphisms.

The article by Giulietti *et al.* describes the methodology to quantify cytokine gene expression using real-time PCR. As this particular class of genes is important in many aspects of immunology, the reader will find this article most helpful, especially the extensive table of primers and probes used to amplify and detect a number of cytokine and housekeeping genes. As is true of most methods, the assay described to quantify cytokine gene expression can be extrapolated to detect many different classes of mRNA. Also included in this article is an excellent overview of the real-time quantitative PCR technology, including a discussion of the available probe chemistries and instruments. It is recommended that all newcomers to the field read this article first.

For routine quantification of DNA or RNA, two general methods of data analysis exist: absolute and relative quantification. Absolute quantification determines the exact template copy number, usually by relating the PCR signal to a standard curve. A discussion of absolute quantification as it applies to the analysis of viral load is included in the article by Niesters. Relative quantification presents the change in expression relative to another sample such as an untreated control. Often, presentation of relative changes in gene expression rather than the absolute degree of change will suffice. Furthermore, relative gene expression assays are less time consuming than absolute quantification methods because standard curves are not necessary.

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For those who wish to present their data as the relative expression, the equations described in the article by Livak and Schmittgen will be helpful in the analysis. This article describes the derivation, assumptions, and examples of the $2^{-\Delta\Delta C_T}$ method of relative gene expression. Also discussed in the article are two variations of the $2^{-\Delta\Delta C_T}$ method as well as some general tips on data analysis including spreadsheet design. The article by Lehmann and Kreipe describes how the $2^{-\Delta\Delta C_T}$ method may be used to analyze data from a quantitative study using specimens from patients.

cDNA microarray and differential display PCR are two leading high-throughput technologies to screen for changes in gene expression. A shortcoming of these technologies is that they are qualitative, not quantitative. Therefore, validation of the change in differential expression should be performed using a quantitative assay. Real-time PCR is ideally suited for this purpose. The article by Rajeevan *et al.* describes a real-time PCR assay that uses SYBR green detection and product melting curve analysis to validate the results from cDNA array and differential display PCR technologies. Comparing the relative gene expression identified by both real-time and high throughput assays will determine if the gene is truly differentially expressed. The use of SYBR green detection rather than TaqMan probes allows the flexibility to quickly validate numerous genes identified by the high-throughput screen.

Another example of how real-time PCR chemistry and instrumentation have enhanced previously used detection schemes is the detection of DNA methylation patterns. Methylation of cytosine within CpG islands to form 5-methylcytosine has been linked to a number of human diseases, in particular, cancer. Laird and co-workers describe the technology referred to as MethyLight. MethyLight is a TaqMan probe-based methylation-specific, real-time PCR assay. Prior to amplification, the DNA is treated with sodium bisulfite to convert unmethylated cytosines to uridine while leaving methylated cytosines intact. Gene-specific primers and/or TaqMan probes are used to discriminate between methylated and unmethylated DNA. As is true of other real-time PCR assays, MethyLight obviates the need for gel electrophoresis, thereby increasing sensitivity and throughput. The sensitivity of MethyLight was recently demonstrated in a study that detected aberrant DNA methylation patterns of esophageal cancer cells that were shed into the plasma of cancer patients (6).

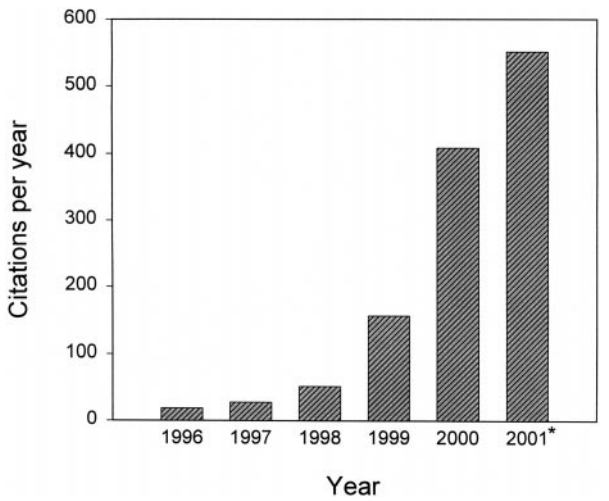

FIG. 1. A Medline search was conducted using the keywords *PCR* and *real-time* or *TaqMan* between the years 1996 and the present. The numbers of citations per year containing these keywords are shown. *Through October, 18, 2001.

The ability to discriminate alleles that harbor a mutation has widespread applications in genetic analyses. Sevall describes a straightforward method of applying real-time PCR for allelic discrimination. TaqMan probes with different fluorescent reporters are designed to hybridize to either the wild-type or mutant gene. Since the efficiency of probe hybridization and subsequent cleavage is dependent on hybridization, an increase in fluorescence signal of one dye over the other indicates homozygosity, whereas an increase in both signals indicates heterozygosity. Real-time PCR data are plotted on an *xy* scatterplot to identify the specific genotype. With this approach, data from 96 samples may be easily genotyped in less than 2 h.

Another important area for quantitative analysis of nucleic acids is in the area of archival biopsies. Pathology departments around the world have backlogged extensive banks of formalin-fixed and paraffin-embedded (FFPE) biopsies. Past issues that have hampered nucleic acid analysis from FFPE tissues include difficulties in extracting intact nucleic acids from FFPE biopsies (especially RNA) and difficulties in isolating specific portions of tissue within the biopsy (e.g., cancerous tissue in an area of normal tissue). As discussed by Lehmann and Kreipe, laser-assisted microdissection coupled with real-time PCR has greatly improved the ability to perform routine analysis on FFPE biopsies. The article discusses how differences in fixation conditions used in various laboratories will impact the ability to amplify nucleic acids. The reader will find helpful protocols on topics ranging from composition of fixatives to optimal fixation conditions and protocols for laser-assisted microdissection. Also discussed is a protocol for the analysis of DNA methylation from FFPE biopsies.

Real-time PCR has not only enhanced our ability to

perform routine quantitative analysis but also has refined high-throughput screening capabilities. A case-in-point is the methodology that combines real-time PCR and molecular beacons to detect single-nucleotide polymorphisms (SNPs). Detection of SNPs may be important in determining an individual's susceptibility to various diseases or how he or she may respond to a particular drug. Readers of the article by Mhlanga and Malmberg will find an excellent overview of both the technology and methodology for real-time detection of SNPs using molecular beacons. Molecular beacons are dual-labeled fluorogenic probes consisting of two regions: a loop region that hybridizes to the target and a self-complementary stem. Fluorescence is quenched until the loop region hybridizes to the target. Once sequence information about the SNP is known, high-throughput SNP detection may be easily and accurately applied using this technology. Protocols and methodologies on primer/molecular beacon design, reaction conditions, and data analyses are described. An example of allele-specific detection of an estrogen receptor SNP using molecular beacons is included.

The ability to simultaneously detect multiple PCR products within a given reaction (e.g., multiplex PCR) has existed for more than 10 years. In the traditional multiplex PCR assays, the different amplicons were detected by their migration on a gel (7). Wittwer *et al.* describe methods and applications of real-time multiplex PCR. The assays are designed for rapid-cycle PCR using glass capillary tubes and single-labeled, rather than dual-labeled, fluorogenic probes. Multiplexing is achieved by detecting differences in color, temperature, *or* both color and temperature. With existing technology, up to four different colors may be detected during the reaction. Coupling both color and temperature allows for the detection of up to 12 PCR products per reaction. Applications of multiplex real-time PCR for allelic discrimination and mutation screening are included in the article.

Precise and accurate quantification of virus-infected cells in species ranging from plants to animals and humans is clearly an important area. This field is rapidly changing and real-time PCR is a major player in the analysis. Niesters describes the use of real-time PCR to quantify viral load. This article is one of several in the issue that discuss the ability of real-time PCR to perform routine diagnostic procedures. Protocols are given for both DNA and RNA viruses. Also discussed in this article is the so-called (nucleic acid sequence-based amplification) NASBA technology. NASBA is an isothermal RNA amplification reaction that generates an RNA amplicon of the opposite polarity of the input RNA. Molecular beacons are then used to detect the RNA without amplification of background DNA. This assay is particularly useful in quantifying retroviruses. A discussion on standard curves and accuracy is included as is a discussion on the need for international standards for quantitation of viral load from laboratory to laboratory.

Numerous technological advances have been made using PCR since its discovery in the mid-1980s. While quantitative PCR was possible prior to the development of real-time PCR, it required specific training, rigorous testing, and lots and lots of accurate pipetting. The development of real-time PCR has enabled scientists to ask many fundamental and important questions in a relatively straightforward and automated fashion. Although some of the difficulties may still need to be worked out, the foundation has been set for the use of real-time quantitative PCR in routine diagnostic assays. This rapid development within a very short period has created one of those defining moments in technology development where the old protocols placed on some shelf or filed away in some folder are remembered and appreciated but undoubtedly will remain unused.

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Guest Editor

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