A Technique Whose Time Has Come

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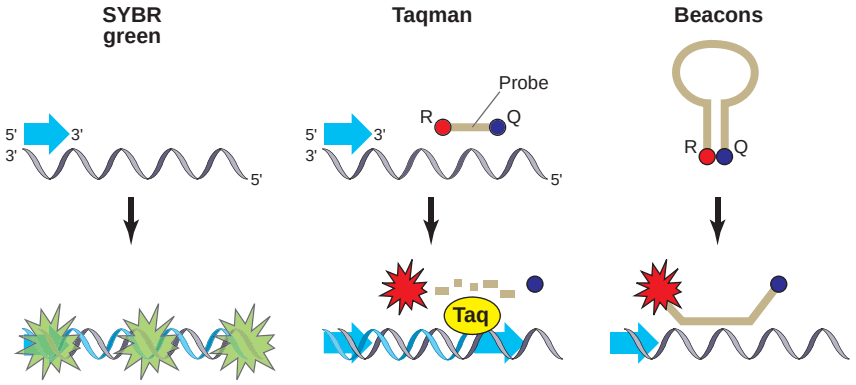
Developed in the mid 1990s for the analysis and quantification of nucleic acids, real-time PCR is a molecular biological technique gaining rapidly in popularity. It is based on the technique of the polymerase chain reaction (PCR) that was first envisioned by Kary Mullis almost 20 years ago, during a moonlit drive through the redwood hills of California (*1*). The technology of PCR (*2*) has become one of the most influential discoveries of the molecular biology revolution and one for which Mullis received the Nobel Prize in 1993. Because of the impact of PCR and the thermostable *Taq* DNA polymerase (the enzyme responsible for the PCR revolution), the pair was named as the first "Molecule of the Year" by *Science* in 1989 (*3*). In many ways, the recent development of real-time PCR seems set to change the general use of PCR.

The advancement provided by the real-time version of PCR is due to its unique ability to monitor the complete DNA amplification process. During conventional PCR, the two strands of a DNA molecule are subjected to a series of heating and cooling cycles that result in DNA strand separation, oligonucleotide primer annealing, and thermostable *Taq* DNA polymerase–directed primer extension, ultimately generating two identical daughter strands. Iterative cycling of the process exponentially amplifies the number of original DNA molecules, hence the term PCR (*4*). After completion of the PCR reaction, amplification products are analyzed by size-fractionation of the amplified sample with the use of gel electrophoresis.

In the mid 1990s, researchers showed that the 5′ nuclease activity of the *Taq* DNA polymerase could be exploited as a method to indirectly assess the level of DNA amplification with the use of specific fluorescent probes (*5*), eliminating the need for electrophoresis. Around the same time, researchers showed that real-time monitoring of the DNA amplification within the PCR reaction tube during the PCR could be achieved by using fluorescent DNA binding dyes, which is known as kinetic PCR (*6*). The coupling of these two processes (*7*, *8*) led to today's technology of fluorescence detection real-time PCR.

In general, analysis of amplification during real-time PCR has been achieved by detecting the fluorescence that is either directly or indirectly associated with the accumulation of the newly amplified DNA (see figure, above). The detection system that is almost synonymous with real-time PCR is the "Taqman" system (*8*), which uses a fluorescence resonance energy transfer (FRET) probe as a reporter system. A FRET probe is a short oligonucleotide that is complementary to one of the strands. The probe contains a "reporter" and a "quencher" fluorescent molecule at the 5′ and 3′ end of the probe, respectively. This probe is included in the real-time PCR reaction along with the required forward and reverse PCR primers. The quencher fluorochrome on the probe, because it is in such close proximity to the reporter, is able to quench the fluorescence of the reporter. As the *Taq* DNA polymerase enzyme replicates the new strand of DNA, the nuclease activity degrades the FRET probe at the 5′ end, which is bound to template DNA strand, in a manner much like the PacMan video game character. This degradation releases the reporter fluorochrome from its proximity to the quencher, resulting in fluorescence of the reporter. Accumulation of fluorescent reporter molecules, as a result of amplification of the target, can then be detected by an appropriate optical sensing system such as the Taqman, an "indirect" system that detects the accumulation of fluorescence rather than the amplified DNA itself. In contrast, a commonly used "direct" method uses a fluorescent DNA (SYBR green) that binds nonspecifically to double-stranded DNA, and the accumulation of the fluorescence bound to the amplified DNA target is measured. The "Molecular Beacon" technology is another direct approach that uses FRET-based fluorescent probes to bind the amplified DNA. In the unbound state, the quencher and reporter fluorochromes are maintained in close proximity via a hairpin loop designed into the sequence of the probe. Binding of the probe at a target sequence–specific region to its complementary strand on the amplified target DNA separates the two fluorochromes, thereby alleviating the FRET interference and allowing the reporter to fluoresce. Related systems that use FRET-based PCR primers incorporated into the amplified DNA have also been developed (*9*). In these systems, the reporter and quencher fluorochromes are maintained in a hairpin loop structure via a sequence that is added to the 5′ end of one of the PCR primers. Disruption of the hairpin loop structure during incorporation of the primer into the amplified DNA product results in loss of the FRET interference, leading to fluorescence of the reporter molecule. Choosing a detection system is a major consideration in developing a real-time PCR assay, and each type of system has its pros and cons. The decision is often a compromise between desired specificity, assay development time, and cost per assay.



Detection systems. Fluorescent detection of amplification can be achieved using double-stranded DNA binding dyes like SYBR green (green starbursts) or with FRET-based probes such as Taqman 5′nuclease-sensitive probes or DNA binding probes (red starbursts).

An inherent property of PCR that is exploited in real-time PCR is that the more copies of nucleic acid one starts with, the fewer cycles of template amplification it takes to make a specific number of

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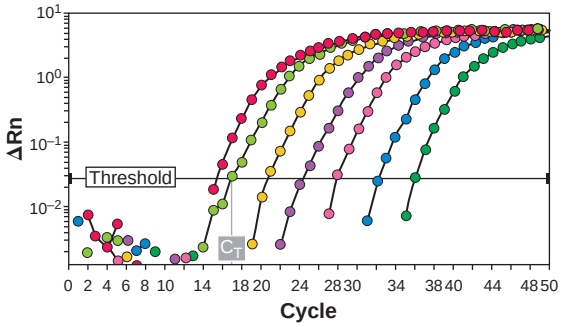
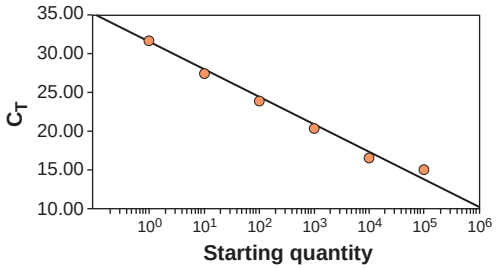
products. Therefore, the number of cycles needed for the amplification-associated fluorescence to reach a specific threshold level of detection (the C_T value) is inversely correlated to the amount of nucleic acid that was in the original sample (see figure, top). Because the progress of amplification is monitored throughout the PCR process in real-time PCR, a C_T value can be determined during the exponential phase of a PCR reaction, when amplification is most efficient and least affected by reaction-limiting conditions. The quantity of DNA in the sample can then be obtained by interpolation of its C_T value versus a linear standard curve of C_T values obtained from a serially diluted standard solution (see figure, right). In practice, such curves are linear over more than five orders of magnitude, a dynamic range that is unsurpassed by other methods for quantitative DNA analysis. It is also possible to quantify differences in nucleic acid levels without such curves. For example, during the exponential phase of PCR, the number of DNA strands theoretically doubles during each cycle (assuming amplification is 100% efficient). Consequently, a sample that has twice the number of starting copies compared with another sample would require one less cycle of amplification to generate an equivalent number of product strands. By using the difference in the C_T values for two samples, therefore, one can mathematically determine the relative difference in the level of the nucleic acid of interest of different samples (*10*).



Amplification time for fluorescence detection. Curves representing the cycle-dependent fluorescence associated with amplification of a specific gene product from 0.1 to 100 ng total RNA are shown. The C_T value for each sample is determined from each curve as the cycle at which the fluorescence achieves a specific threshold value. ΔRn, normalized fluorescence.



Dynamic range of real-time PCR. Fluorescent detection of the amplification of the gene product is linear over five orders of magnitude. Values were calculated on the basis of C_T values from amplification figure. Slope, –3.538; *y* intercept, 31.806; correlation coefficient, 0.991.

Instrumentation systems for real-time PCR have undergone extensive changes already. Initially they were large machines that took up almost an entire lab bench and were incapable of a true analysis in real time. As the technology improved, systems became available that allow the PCR reaction to be monitored as it occurs. The size and cost of these systems has been reduced so dramatically that they are now reasonably priced units with very small footprints. Given the added capabilities of the real-time technology, it is likely that in the near future such systems will become the standard PCR platform in the general laboratory, in much the same way that the 96- and 384-microwell PCR machines have superseded initial large-tube format units.

In the laboratory, real-time reverse transcriptase–PCR (RT-PCR) has become the method of choice for the rapid and quantitative examination of the expression of specific genes. Users of the method can rapidly, reproducibly, and statistically determine even small (twofold) changes in the expression of hundreds of samples per day. Such analyses would be very difficult and laborious with the use of traditional hybridization techniques, such as Northern blotting or RNase protection assays. The low RNA quantities required (nanograms) make this assay more suitable for the analysis of samples obtained from laser microdissected tissue. In addition, real-time approaches are perfectly adaptable for high-throughput and quantitative gene expression studies, especially with the recent development of high-throughput 384-well real-time PCR instruments. These advances are timely, given the completion of the Human Genome Project. Recent advances in microtechnologies, such as DNA chip technology, coupled with proteomics and metabonomics (*11*) are establishing the new science of systems biology (*12*). This division of biology seeks to use information from the integrated analysis of genes or proteins and metabolism to develop computational models of cellular function and physiology. Such models are enhanced through the availability of quantitative information on the expression of genes, proteins, or metabolites (*13*). Quantitative real-time PCR analysis of gene expression is likely to play a key role in this burgeoning field.

Though PCR has influenced drastically the way in which molecular biological research is conducted, its impact on our everyday lives has yet to be fully realized. Because of its reduced detection times and simplification of quantitation, real-time PCR systems are likely to have the greatest impact on the general public in environmental monitoring and nucleic acid diagnostics. Most uses of PCR-based diagnostics still require specialized laboratory services, but several companies are developing devices that will be to be able to allow such rapid PCR analysis to be performed in the near future at the point the sample is collected instead of in a far-removed laboratory. Devices coupling microfluid technology with real-time PCR analysis of nucleic acid detection are set to make a huge impact in many facets of our lives as we enter the genomic information era (*14*). Such devices would incorporate nucleic acid extraction and PCR detection in small, disposable systems that are readable through attachments to a personal computer. This technology could potentially enable in-home testing for nucleic acids from bacterial or viral pathogens. As methods for the quantitative analysis of gene expression and DNA levels continue to evolve in sophistication, devices that incorporate the concepts of real-time PCR will likely herald the era of individualized genomics and genetic testing.

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