

An Introduction to Real-Time PCR

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Abstract

The development of instruments that allowed real-time monitoring of fluorescence within PCR reaction vessels was a very significant advance. The technology is very flexible and many alternative instruments and fluorescent probe systems have been developed and are currently available. Real-time PCR assays can be completed very rapidly since no manipulations are required post-amplification. Identification of the amplification products by probe detection in real-time is highly accurate compared with size analysis on gels. Analysis of the progress of the reaction allows accurate quantification of the target sequence over a very wide dynamic range, provided suitable standards are available. Further investigation of the real-time PCR products within the original reaction mixture using probes and melting analysis can detect sequence variants including single base mutations.

Since the first practical demonstration of the concept real-time PCR has found applications in many branches of biological science. Applications include gene expression analysis, the diagnosis of infectious disease and human genetic testing.

Due to their capability in fluorimetry the real-time machines are also compatible with alternative amplification methods such as NASBA provided a fluorescence end-point is available.

PCR: The Early Years

The theoretical concept of producing many copies of a specific DNA molecule by a cycling process using DNA polymerase and oligonucleotide primers was first expounded in a paper by Kleppe and colleagues in 1971 (Kleppe *et al.*, 1971). At that time, the practical exploitation of such a process must have seemed remote to the biologists who read the paper. This was due to the difficulty and cost of producing oligonucleotides, the non-availability of thermostable DNA polymerases and the lack of automated thermocycling instruments. By the time of the first demonstration of the PCR process by Saiki and colleagues in 1985 (Saiki *et al.*, 1985) automated oligonucleotide synthesisers were commonly available. This meant that the potential of PCR in a wide range of applications was recognised. However, it was still necessary to inject fresh thermo-labile polymerase prior to each elongation step and thermal cyclers were still in development. Consequently, the key step in realising the potential of the PCR was probably the use of a thermostable polymerase which was first described in 1988 (Saiki *et al.*, 1988). Since the first description of a practical DNA amplification process many refinements have been described and automatic thermal cyclers have become standard laboratory equipment. PCR is now an essential tool for many biologists and the standard protocols are very simple and user friendly. The exponential amplification process provides nanogram quantities of essentially identical DNA molecules starting from a few copies of a target sequence. The amplified material (the PCR amplicon) is available in sufficient quantity to be identified by size analysis, sequencing or by probe hybridisation. It can also be cloned readily or used as a reagent.

The Need for Real-Time PCR

Much of the technical effort involved in standard PCR is now directed toward positive recognition of the amplicons. The important methods of post-PCR analysis rely on either the size or sequence of the amplicon. Gel electrophoresis is often used to measure the size of the amplicon and this is both inexpensive and simple to implement. Unfortunately, size analysis has limited specificity since different molecules of approximately the same molecular weight cannot be distinguished. Consequently, gel electrophoresis alone is not a sufficient PCR end-point in many instances, including most clinical applications. Characterisation of the product by its sequence is far more reliable and informative. Probe hybridisation assays for this purpose are available but many are multi-step procedures. Such methods are time-consuming and care must be taken to ensure that amplicons accidentally released into the laboratory environment do not contaminate the DNA preparation and clean rooms.

Real-time PCR machines greatly simplify amplicon recognition by providing the means to monitor the accumulation of specific products continuously during cycling. All current instruments designed for real-time PCR measure the progress of amplification by monitoring changes in fluorescence within the PCR tube. Changes in fluorescence can be linked to product accumulation by a variety of methods. A further advantage of the real-time format is that the analysis can be performed without opening the tube which can then be disposed of without the risk of dissemination of PCR amplicons or other target molecules into the laboratory environment. Although alternative methods for avoiding PCR contamination are available, containment within the PCR vessel is likely to be the most efficient and cost-effective. A major drawback of standard PCR formats that rely on end-point analysis is that they are not quantitative because the final yield of product is not primarily dependent upon the concentration of the target sequence in the sample. Real-time PCR overcomes this limitation.

Real-Time PCR Chemistries

There are two general approaches used to obtain a fluorescent signal from the synthesis of product in PCR. The first depends upon the property of fluorescent dyes such as SYBR Green I to bind to double stranded DNA and undergo a conformational change that result in an increase in their fluorescence. The second approach is to use fluorescent resonance energy transfer (FRET). These methods use a variety of means to alter the relative spatial arrangement of photon donor and acceptor molecules. These molecules are attached to probes, primers or the PCR product and are usually selected so that amplification of a specific DNA sequence brings about an increase in fluorescence at a particular wavelength.

A major advantage of the real-time PCR instruments and signal transduction systems currently available is that it is possible to characterise the PCR amplicon *in situ* on the machine. This is done by analysis of the melting temperature and/or probe hybridisation characteristics of the amplicon within the PCR reaction mixture. In the intercalating dye system the melting temperature of the amplicon can be estimated by measuring the level of fluorescence emitted by the dye as the temperature is increased from below to above the expected melting temperature. The methods that rely upon probe hybridisation to produce a fluorescent signal are generally less liable to produce false positive results than alternative methods such as the use of intercalating dyes to detect net synthesis of double stranded DNA (dsDNA) followed by melting analysis of the product. Hybridisation, ResonSense and hydrolysis probe systems give fluorescent signals that are only produced when the target sequence is amplified and are unlikely to give false positive results. An additional feature of the hybridisation, ResonSense and related methods is that it is also possible to measure the temperature at which the probes disassociate from their complementary sequences giving further verification of the specificity of the amplification reaction. An important feature of many of the probe systems is that they are compatible with multiplexing due to the availability of fluorophores with resolvable emission spectra. The chemistries available are discussed in detail in Chapter 3.

Real-Time PCR Instrumentation

Thermal cyclers with integrated fluorimeters and some arrangement for transferring excitation light from a source into the reaction vessel and then from the sample to a detector are required for real-time PCR. The heating blocks that are the mainstay of the standard PCR instrument market present several technical challenges in conversion to application in real-time machines. The main problem being that the light must be channelled through the lid of the block and the cap of the reaction vessel across an air gap and then into the sample. Emitted light must then take the return path. Although blocks are used by several real-time machines including the first commercial instrument (ABI 7700), the difficulties associated with them have led to the development of alternative designs. The LightCycler[®] (LC24) was the forerunner of machines that use air as the heating/cooling medium. Thermal transfer via air has the advantage of greater uniformity and rapidity than can be achieved on block-based cyclers, besides allowing shortening of the light path. Besides differing in the choice of heating medium real-time PCR machines also provide a range of options for the light source and detection of fluorescence. Current machines tend to allow the excitation and detection of multiple dyes so that internal standards and multiplex reactions are possible. There is also a tendency to build in a bias toward the use of either universal donor or universal recipient chemistry (see Chapter 3).

Since their introduction, the cost of real-time PCR instruments has fallen in tandem with continual improvement in their capability and accuracy. This has been the result of competition, the volume of sales and the introduction into the marketplace of improved designs dependent on new technology. These trends are unlikely to be reversed and will contribute to the growth in real-time PCR's popularity. Instrumentation for real-time PCR is described and discussed in more detail in Chapter 2.

Quantification

Unlike standard PCR, real-time PCR instruments measure the kinetics of product accumulation in each PCR reaction tube. Generally, no product is detected during the first few temperature cycles as the fluorescent signal is below the detection threshold of the instrument. However, most combinations of machine and fluorescence reporter are capable of detecting the accumulation of amplicons before the end of the exponential amplification phase. During this time the efficiency of PCR is often close to 100% giving a doubling of the quantity of product at each cycle. As product concentrations approach the nanogram per μl level the efficiency of amplification falls primarily because the amplicons re-associate during the annealing step. This leads to a phase during which the accumulation of product is approximately linear with a constant level of net synthesis at each cycle. Finally, a plateau is reached when net synthesis approximates zero. Quantification in real-time PCR is done by measuring the number of cycles required for the fluorescent signal to reach a threshold level or the second derivative maximum of the fluorescence versus cycle curve. This cycle number is proportional to the number of copies of template in the sample. Real-time quantification is discussed further in Chapters 6 and 7.

SNP Detection

The methods used to verify the identity of the amplicon(s) produced in real-time PCR are also often sufficiently powerful to detect small variations between sequences. Variations in sequence ranging from single nucleotide polymorphisms (SNPs) have been successfully identified in real-time PCR assays.

One common approach to the detection of sequence variation is to compare melting curves. In general, the effect of base substitutions on the melting kinetics of PCR products is too small to be detected reliably (if at all). However, one group (Wittwer *et al.*, 2003) has demonstrated that heteroduplexes of relatively long amplicons differing by a SNP can be distinguished from the homoduplexes on the basis of their melting curves. This was presented as the basis of a method

for mutation screening. More commonly, the melting curves of short fluorescent probes are used to distinguish between amplicons, for example (Edwards *et al.*, 2001b; Whalley *et al.*, 2001). This method is sensitive to SNPs, which usually cause a shift in the melting peak of several degrees. A common alternative to the melting curve approach is to use hydrolysis (TaqMan) probes. The efficiency of the 5'-3' endonuclease reaction is greatly impaired when a well-designed probe mismatches its target sequence by even a single base. The detection of mutations by real-time PCR is discussed in Chapter 8. Although the melting curve and hydrolysis probe methods for mutation analysis are widely used they are only able to detect sequences that represent a large proportion of the population. The quantitative real-time ARMS assay described in Chapter 9 is designed to detect the emergence of significant sequence mutants within a background that remains mainly of the parent type.

Real-Time PCR Data Analysis

The software provided with real-time PCR instruments allows three principle types of data analysis. 1) Measurement of the cycle number at which any increase in the fluorescence within each reaction vessel reaches significance. 2) The data are used in conjunction with the results from external standards to estimate the original number of template copies. 3) Melting curves are transformed to provide plots of $-dF/dT$ against T (F = fluorescence and T =temperature) in which a peak (melting peak) occurs at the equilibrium temperature for each duplex. In general the different software is easy to use and allows rapid and reproducible data analysis.

Non-PCR Applications

Real-time PCR machines are also capable of use as real-time fluorimeters. For example, one simple application is estimation of the melting temperature (T_m) of an oligonucleotide. The oligonucleotide is mixed with its complementary sequence in the presence of a dye such as SYBR Green I, the temperature is increased and the level of

fluorescence is measured to give a melting curve from which the T_m may be deduced.

Chapter 10 presents an alternative application using a real-time PCR instrument that relies on real-time fluorimetry. NASBA is a method for the isothermal amplification of RNA that produces quantities of antisense RNA copies. Molecular beacons complementary to the product are used to give a fluorescent signal.

The Growth in the Use of Real-Time PCR

In a relatively short time since their first introduction in the mid-1990s real-time PCR machines have become widely available to biologists. This has led to an explosion in the number of publications describing applications of the method. Indeed, a graph of number of papers against time resembles a real-time PCR plot (Figure 1). Most of the main applications that exploit real-time PCR previously relied on standard PCR and the main fields included diagnostic microbiology and human genetic analysis. However, the decreased hands-on time, increased reliability and improved quantitative accuracy of real-time PCR methods are contributing to a widening of their use into areas that were not previously dominated by PCR. For example, it has been

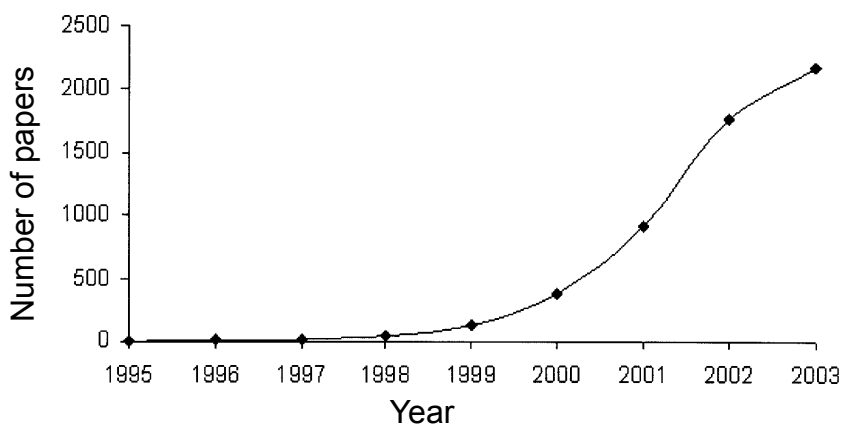


Figure 1. PubMed at NCBI (<http://www.ncbi.nlm.nih.gov/>) was searched by year for the term 'real-time PCR'. The result for 2003 was for an incomplete year.

exploited for gene expression analysis (Edwards and Saunders, 2001; Sabersheikh and Saunders, 2003).

Applications of Real-Time PCR

In recent years real-time PCR has found many biological applications. These can usually be classified as either quantitative or qualitative methods and according to whether the probes are used to distinguish between sequence variants or simply as reporters.

The simplest application of real-time PCR is for the detection of specific gene sequences within a complex mixture. Such assays are useful in the diagnosis of infectious disease where assays for DNA sequences specific for a wide range of pathogens have been developed. Several such applications are discussed in Chapters 11 and 12.

An important application of quantitative real-time PCR is the measurement of RNA transcript levels to assess gene expression. This application is making a critical contribution to our understanding of the interplay of host and microbe responses during infection. Gene expression studies can also help us to understand the functioning of normal tissues and to elucidate the pathogenesis of non-infectious diseases. The analysis of mRNA expression is discussed in Chapter 7. Other important applications of quantitative real-time PCR include the assessment of gene ratios in tumour tissues (Lehmann *et al.*, 2000; Kim *et al.*, 2002) and the measurement of pathogen numbers in clinical specimens (Brechtbuehl *et al.*, 2001; Eishi *et al.*, 2002).

Real-time PCR is frequently used for genotyping humans and human pathogens. In certain epidemiological studies the target sequences may be anonymous markers selected on the strength of their ability to discriminate between individuals or their linkage with particular phenotypes. More frequently target sequences are employed that are directly associated with a particular phenotype. Real-time PCR assays that use oligonucleotide probes for the detection of SNPs are now widely used. Examples include assays for bacterial identification (Edwards *et al.*, 2001a; Logan *et al.*, 2001), to detection of drug resistance (Edwards

et al., 2001b; Whalley *et al.*, 2001) and for diagnosis of human genetic disease (Costa *et al.*, 2003; Vrettou *et al.*, 2003).

References

- Brechtbuehl, K., Whalley, S. A., Dusheiko, G. M., and Saunders, N. A. 2001. A rapid real-time quantitative polymerase chain reaction for hepatitis B virus. *J. Virol. Methods* 93: 105-113.
- Costa, C., Pissard, S., Girodon, E., Huot, D., and Goossens, M. 2003. A one-step real-time PCR assay for rapid prenatal diagnosis of sickle cell disease and detection of maternal contamination. *Mol. Diagn.* 7: 45-48.
- Edwards, K. J., Kaufmann, M. E., and Saunders, N. A. 2001a. Rapid and accurate identification of coagulase-negative staphylococci by real-time PCR. *J. Clin. Microbiol.* 39: 3047-3051.
- Edwards, K. J., Metherell, L. A., Yates, M., and Saunders, N. A. 2001b. Detection of *rpoB* mutations in *Mycobacterium tuberculosis* by biprobe analysis. *J. Clin. Microbiol.* 39: 3350-3352.
- Edwards, K. J., and Saunders, N. A. 2001. Real-time PCR used to measure stress-induced changes in the expression of the genes of the alginate pathway of *Pseudomonas aeruginosa*. *J. Appl. Microbiol.* 91: 29-37.
- Eishi, Y., Suga, M., Ishige, I., Kobayashi, D., Yamada, T., Takemura, T., Takizawa, T., Koike, M., Kudoh, S., Costabel, U., Guzman, J., Rizzato, G., Gambacorta, M., du Bois, R., Nicholson, A. G., Sharma, O. P., and Ando, M. 2002. Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis. *J. Clin. Microbiol.* 40: 198-204.
- Kim, Y. R., Choi, J. R., Song, K. S., Chong, W. H., and Lee, H. D. 2002. Evaluation of HER2/neu status by real-time quantitative PCR in breast cancer. *Yonsei Med. J.* 43: 335-340.
- Kleppe, K., Ohtsuka, E., Kleppe, R., Molineux, I., and Khorana, H. G. 1971. Studies on polynucleotides. XCVI. Repair replications of short synthetic DNA's as catalyzed by DNA polymerases. *J. Mol.*

Biol. 56: 341-361.

Lehmann, U., Glockner, S., Kleeberger, W., von Wasielewski, H. F., and Kreipe, H. 2000. Detection of gene amplification in archival breast cancer specimens by laser-assisted microdissection and quantitative real-time polymerase chain reaction. *Am. J. Pathol.* 156: 1855-1864.

Logan, J. M., Edwards, K. J., Saunders, N. A., and Stanley, J. 2001. Rapid identification of *Campylobacter* spp. by melting peak analysis of biprobes in real-time PCR. *J. Clin. Microbiol.* 39: 2227-2232.

Sabersheikh, S., and Saunders, N. A. 2003. Quantification of virulence-associated gene transcripts in epidemic methicillin resistant *Staphylococcus aureus* by real-time PCR. *Mol. Cell. Probes* In press.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230: 1350-1354.

Vrettou, C., Traeger-Synodinos, J., Tzetis, M., Malamis, G., and Kanavakis, E. 2003. Rapid screening of multiple beta-globin gene mutations by real-time PCR on the LightCycler: application to carrier screening and prenatal diagnosis of thalassemia syndromes. *Clin. Chem.* 49: 769-776.

Whalley, S. A., Brown, D., Teo, C. G., Dusheiko, G. M., and Saunders, N. A. 2001. Monitoring the emergence of hepatitis B virus polymerase gene variants during lamivudine therapy using the LightCycler. *J. Clin. Microbiol.* 39: 1456-1459.

Wittwer, C. T., Reed, G. H., Gundry, C. N., Vandersteen, J. G., and Pryor, R. J. 2003. High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin. Chem.* 49: 853-860.

