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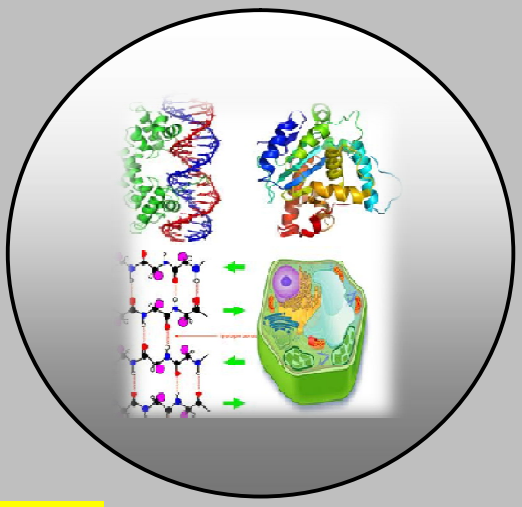

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RESEARCH PAPER

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Biological Investigation by Polymerase Chain Reaction (PCR)

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ABSTRACT

Polymerase chain reaction (PCR) enables researchers to produce millions of copies of a specific DNA sequence in approximately two hours. This automated process bypasses the need to use bacteria for amplifying DNA. *Real-time polymerase chain reaction (PCR) has emerged as a robust and widely used methodology for biological investigation because it can detect and quantify very small amounts of specific nucleic acid sequences. As a research tool, a major application of this technology is the rapid and accurate assessment of changes in gene expression as a result of physiology, pathophysiology, or development. This method can be applied to model systems to measure responses to experimental stimuli and to gain insight into potential changes in protein level and function. Thus physiology can be correlated with molecular events to gain a better understanding of biological processes. For clinical molecular diagnostics, real-time PCR can be used to measure viral or bacterial loads or evaluate cancer status. Here, we discuss the basic concepts, chemistries, and instrumentation of real-time PCR and include present applications and future perspectives for this technology in biomedical sciences and in life science education*

Key words: PCR, Biological Investigation, Molecular Diagnostics, and DNA

INTRODUCTION

TIME POLYMERASE CHAIN REACTION (PCR) is based on the revolutionary method of PCR, developed by Kary Mullis in the 1980s, which allows researchers to amplify specific pieces of DNA more than a billion-fold (Mullis 1990. Mullis and Faloona 1987. Saiki et al. 1985). PCR-based strategies have propelled molecular biology forward by enabling researchers to manipulate DNA more easily, thereby facilitating both common procedures, such as cloning, and huge endeavors such as the Human Genome Project (Ausubel et al. 2005, Olson 1989). Real-time PCR represents yet another technological leap forward that has opened up new and powerful applications for researchers throughout the world.

This is in part because the enormous sensitivity of PCR has been coupled to the precision afforded by "real-time" monitoring of PCR products as they are generated.

Higuchi and co-workers (Higuchi et al. 2001, Higuchi et al 1992) at Roche Molecular Systems and Chiron accomplished the first demonstration of real-time PCR. By including a common fluorescent dye called ethidium bromide (EtBr) in the PCR and running the reaction under ultraviolet light, which causes EtBr to fluoresce, they could visualize and record the accumulation of DNA with a video camera. It has been known since 1966 that EtBr increases its fluorescence upon binding of nucleic acids (Le Pecq and Paoletti 1966), but only by combining this fluorescent chemistry with PCR and real-time videography could real-time PCR be born as it was in the early 1990s. Subsequently, this technology quickly matured into a competitive market, becoming commercially widespread and scientifically influential. This is evidenced by both the number of companies offering real-time PCR instrumentation (and reagents) and the rapidly increasing numbers of scientific publications pertaining to real-time PCR. Real-time PCR instrumentation was first made commercially available by Applied Biosystems in 1996, after which several other companies added new machines to the market. Presently, Applied Biosystems, BioGene, Bioneer, Bio-Rad, Cepheid, Corbett Research, Idaho Technology, MJ Research, Roche Applied Science, and Stratagene all offer instrumentation lines for real-time PCR. A significant portion of these machines are used for academic research, and, according to a 2003 survey of 406 scientists, 48% projected that they would increase the number of amplifications performed in the coming year (BioInformatics 2003). Increased usage of real-time PCR has translated into scientific publications. Figure 1 shows the number of publications in the Medline database that contain the words "real-time" and "PCR" or "real-time" and "polymerase chain reaction" in their title or abstract. In 2004, there were 3,522 such publications, representing 43% growth over 2003, in which there were 2,462 publications. The impact of real-time PCR technology on scientific literature is likely to be greater than these numbers imply, as they represent only a fraction of the total number of studies that utilize real-time PCR in their methods. Thus real-time PCR expands the influence of PCR-based innovations and presents intriguing directions for the future of biomedical sciences (especially molecular diagnostics and molecular physiology) and life science education (Lederberg 1953, Walker 2002).

Widespread use has also resulted in a multiplicity of names for the technology, each with a different shade of meaning. Real-time PCR simply refers to amplification of DNA (by PCR) that is monitored while the amplification is occurring. The benefit of this real-time capability is that it allows the researcher to better determine the amount of starting DNA in the sample before the amplification by PCR. Present day real-time methods generally involve fluorogenic probes that "light up" to show the amount of DNA present at each cycle of PCR. "Kinetic PCR" refers to this process as well. "Quantitative PCR" refers to the ability to quantify the starting amount of a specific sequence of DNA. This term predates real-time PCR because it can refer to any PCR procedure, including earlier gel-based end-point assays, that attempts to quantify the starting amount of nucleic acid.

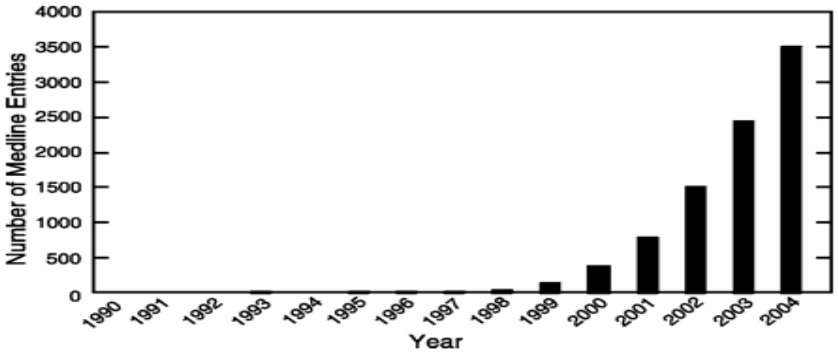

Fig 1. Growing use of real-time PCR. Shown are the numbers of publications in the Medline database that contain the words "real- time" and "PCR" or "real-time" and "polymerase chain reaction" in their title or abstract for the years of 1990–2004

Rarely, one might see the term "quantitative fluorescent PCR" to designate that the quantification was accomplished via measuring output from a fluorogenic probe, although this is redundant because all of the present chemistries for real-time PCR are fluorescent. In addition, if reverse transcriptase enzymes (see below) are used before PCR amplification in any of the above situations, then "RT-PCR" replaces "PCR" in the term. Today, the two most common terms, real-time and quantitative, are often used interchangeably or in combination, because real-time PCR is quickly becoming the method of choice to quantify nucleic acids.

THE GOAL OF REAL-TIME PCR

The basic goal of real-time PCR is to precisely distinguish and measure specific nucleic acid sequences in a sample even if there is only a very small quantity. Real-time PCR amplifies a specific target sequence in a sample then monitors the amplification progress using fluorescent technology. During amplification, how quickly the fluorescent signal reaches a threshold level correlates with the amount of original target sequence, thereby enabling quantification. In addition, the final product can be further characterized by subjecting it to increasing temperatures to determine when the double-stranded product "melts." This melting point is a unique property dependent on product length and nucleotide composition. To accomplish these tasks, conventional PCR has been coupled to state-of-the-art fluorescent chemistries and instrumentation to become real-time PCR.

WHAT IS PCR?

At its core, real-time PCR technology utilizes conventional PCR. PCR is a procedure by which DNA can be copied and amplified (Powledge 2004). As shown in Fig. 2, PCR exploits DNA polymerases to amplify specific pieces of DNA using short, sequence-specific oligonucleotides added to the reaction to act as primers. The first and most commonly used of these enzymes is *Taq* DNA polymerase (from *Thermus aquaticus*), whereas *Pfu* DNA polymerase (from *Pyrococcus furiosus*) is used widely because of its higher fidelity when copying DNA.

Although these enzymes are subtly different, they both have two basic capabilities that make them useful for PCR: *1*) they can generate new strands of DNA using a DNA template and primers, and *2*) they are heat resistant. The latter attribute is necessary because after each round of DNA copying, the resulting double-stranded DNA (dsDNA) must be "melted" into single strands by high temperatures within the reaction tube (~95°C). The reaction is then cooled to allow the oligonucleotide primers to anneal to the now single-stranded template DNA and direct the DNA polymerase enzyme to initiate elongation by adding single complementary nucleotides to create a new complete strand of DNA. Thus dsDNA is created. This new dsDNA must then be melted apart before the next cycle of copying can occur. Therefore, if the reaction works with perfect efficiency, there will be twice as much specific dsDNA after each cycle of PCR. In reality, reactions do not maintain perfect efficiency because reactants within the PCR are consumed after many cycles, and the reaction will reach a plateau (Fig. 3). In addition, self-annealing of the accumulating product may also contribute to the "plateau effect" (Wittwer et al.1997). In fact, it is this very attribute of PCRs that makes real-time PCR technology so necessary. Because the reaction is able to efficiently amplify DNA only up to a certain quantity before the plateau effect, there is no way to reliably calculate the amount of starting DNA by quantifying the amount of product at the completion of the PCR. That is to say, no matter how much of a specific target DNA sequence is present before PCR, there can be similar amounts of amplified DNA after PCR, and any distinct correlation between starting and finishing quantities is lost. Real-time PCR addresses this problem by taking advantage of the fact that DNA amplifications do occur efficiently early in the reaction process and, therefore, measures product formation during this "exponential phase" (Fig. 3). This measurement correlates to the amount of specific starting DNA, thereby allowing quantification.

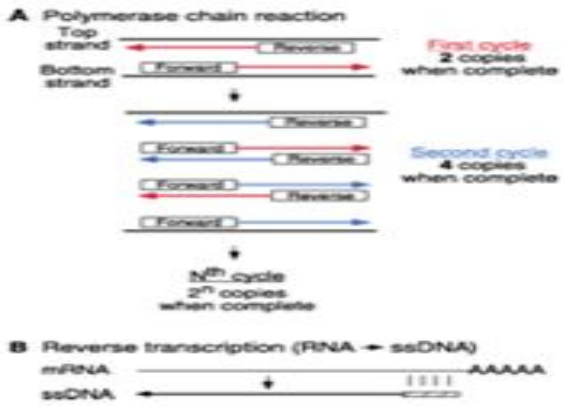

Fig. 2. Enzyme reactions that make real-time PCR possible.

A: PCR is depicted. High temperatures are used to "melt" double-stranded (ds) DNA into its top and bottom strands. This mixture is cooled in the presence of sequence-specific primers (denoted as forward and reverse) that anneal to their targets, and an optimal temperature is then applied to allow elongation of complementary DNA (arrows) by the action of DNA polymerase to complete a cycle.

This is repeated numerous times, and, if no reagents are limiting, 2^n copies of the desired DNA fragment can be obtained. *B*: because DNA polymerase does not utilize RNA as a template, the conversion of RNA to DNA can be achieved using the enzyme reverse transcriptase. ssDNA, single-stranded DNA.

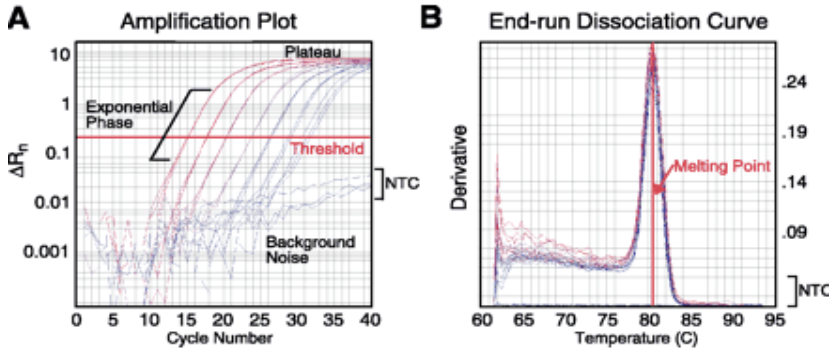

Fig. 3. Typical real-time PCR results.

***A*: amplification plot illustrating the increase in fluorescent reporter signal (*y*-axis, note the log scale) with each PCR cycle (*x*-axis). The *y*-axis units (ΔR_n) actually reflect the reporter signal normalized to a passive reference dye in the reaction buffer. The curves seen with a no-template control (NTC), which lacks added DNA, show that the primers alone do not generate a signal and that the reagents used in this assay showed no DNA contamination. *B*: dissociation curve for this analysis showing a single, sharp peak, suggesting that only a specific PCR product was generated with this set of primers**.

REVERSE TRANSCRIPTASE EXTENDS THE UTILITY OF REAL-TIME PCR

A major limitation of DNA polymerases (and PCR itself) is that they generally must use DNA as their template. They cannot, for example, amplify RNA in a similar manner. This problem can be overcome by another enzyme, reverse transcriptase, which generates complementary DNA (or cDNA) from an RNA template. Reverse transcriptases are enzymes used in nature by retroviruses, including human immunodeficiency virus and hepatitis C virus, to generate DNA from viral RNA. The virus-derived DNA can then be inserted into the host's genome. In the laboratory, reverse transcriptase is used to convert RNA to cDNA that can then be used for multiple purposes including PCR-based applications. There are several commonly used reverse transcriptases, including avian myeloblastosis virus reverse transcriptase, Moloney murine leukemia virus reverse transcriptase, or engineered enzymes that enhance polymerase activity or decrease unwanted nuclease activities (e.g., Omniscript, PowerScript, StrataScript, SuperScript II, etc.). Under the appropriate reaction conditions, the relative amount of a given cDNA generated by reverse transcription is proportional to the relative amount of its RNA template. If this were not the case, measurements of cDNA quantities would have no relevance to RNA. Reliably generated cDNA can be used as the raw material for real-time PCR, thereby utilizing its precision and sensitivity to determine changes in gene expression (i.e., RNA levels). This is called real-time RT-PCR and has become the most popular method of quantitating steady-state mRNA levels (Bustin 2000).

It is most often used for two reasons: either as a primary investigative tool to determine gene expression or as a secondary tool to validate the results of DNA microarrays (BioInformatics 2003). Because of the precision and sensitivity of real-time RT-PCR, even subtle changes in gene expression can be detected. Thus real-time PCR can be used to assess both DNA and RNA levels with great sensitivity and precision.

CHEMISTRY OF REAL-TIME PCR

The key to real-time PCR is the ability to monitor the progress of DNA amplification in real time. This is accomplished by specific chemistries and instrumentation. Generally, chemistries consist of special fluorescent probes in the PCR (Fig. 4). Several types of probes exist, including DNA-binding dyes like EtBr or SYBR green I, hydrolysis probes (5′-nuclease probes), hybridization probes, molecular beacons, sunrise and scorpion primers, and peptide nucleic acid (PNA) light-up probes. Each type of probe has its own unique characteristics, but the strategy for each is simple. They must link a change in fluorescence to amplification of DNA

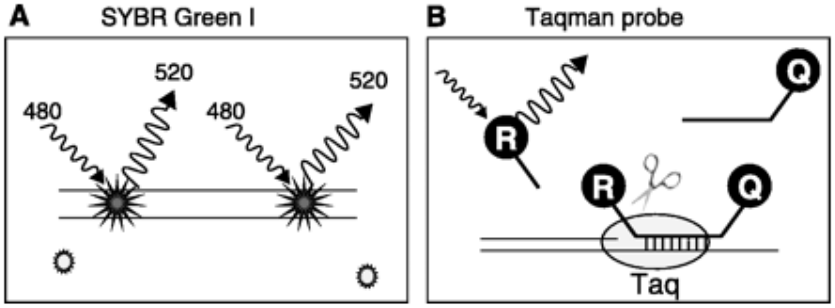

Fig. 4. Real-time PCR chemistries. *A*: SYBR green I fluoresces (absorbing light of 480-nm wavelength and emitting light of 520-nm wavelength) when associated with dsDNA. *B*: other detection formats often utilize compatible fluorphores. Shown in this example is the Taqman probe, which contains a reporter fluorophore (R) that emits at a wavelength absorbed by the quencher fluorophore (Q). During PCR amplification, the DNA polymerase (*Taq*) cleaves the probe, thus liberating the reporter from the quencher and allowing for measurable fluorescence.

SYBR green I bind to the minor groove of dsDNA, emitting 1,000-fold greater fluorescence than when it is free in solution (Fig. 4*A*) (Wittwer 1997). Therefore, the greater the amount of dsDNA presents in the reaction tube, the greater the amount of DNA binding and fluorescent signal from SYBR green I. Thus any amplification of DNA in the reaction tube is measured. Other dsDNA-specific dyes (e.g., BEBO, YOYO-1, TOTO-1, etc.) have also been described but are not as widely used. The primary concern with the usage of any of these sequence-independent dsDNA-binding probes is specificity. To help ensure specificity, the dissociation curve of the amplified product can be analyzed to determine the melting point (Fig. 3). If there are two or more peaks, it suggests that more than one amplified sequence was obtained, and the amplification was not specific for a single DNA target.

Hydrolysis probes (also called 5′-nuclease probes because the 5′-exonuclease activity of DNA polymerase cleaves the probe) offer an alternative approach to the problem of specificity (Fig. Wilson, 1997). These are likely the most widely used fluorogenic probe format (Mackay 2004) and are exemplified by TaqMan probes. In terms of structure, hydrolysis probes are sequence-specific dually fluorophore-labeled DNA oligonucleotides. One fluorophore is termed the quencher and the other is the reporter. When the quencher and reporter are in close proximity, that is, they are both attached to the same short oligonucleotide, the quencher absorbs the signal from the reporter. This is an example of fluorescence resonance energy transfer (also called Förster transfer) in which energy is transferred from a "donor" (the reporter) to an "acceptor" (the quencher) fluorophore. During amplification, the oligonucleotide is broken apart by the action of DNA polymerase (5′-nuclease activity) and the reporter and quencher separate, allowing the reporter's energy and fluorescent signal to be liberated. Thus destruction or hydrolysis of the oligonucleotide results in an increase of reporter signal and corresponds with the specific amplification of DNA. Examples of common quencher fluorophores include TAMRA, DABCYL, and BHQ, whereas reporters are more numerous (e.g., FAM, VIC, NED, etc). Hydrolysis probes afford similar precision as SYBR green I (Wilhelm 2003), but they give greater insurance regarding the specificity because only sequence-specific amplification is measured. In addition, hydrolysis probes allow for simple identification of point mutations within the amplicon using melting curve analysis (see COMMON APPLICATIONS FOR REAL-TIME PCR).

There are several other variations on the reporter-quencher theme, including molecular beacons, sunrise primers, and scorpion primers. They each seek to keep the reporter and quencher together before amplification while separating them and generating the fluorescence signal during amplification. Another class, called hybridization probes, uses donor and acceptor fluorophores, whereas PNAs containing thiazole orange fluorophores (called light-up probes) also emit greater signal upon binding of DNA (Svanvik 2000). These do not represent an exhaustive list, as much other specific and nonspecific chemistry exist. In addition, new fluorescent chemistries are continually being developed with a focus on increasing sensitivity (by increasing the signal-to-noise ratio) and specificity, enhancing multiplexing capabilities, and reducing cost.

THE INSTRUMENTATION OF REAL-TIME PCR

A critical requirement for real-time PCR technology is the ability to detect the fluorescent signal and record the progress of the PCR. Because fluorescent chemistries require both a specific input of energy for excitation and a detection of a particular emission wavelength, the instrumentation must be able to do both simultaneously and at the desired wavelengths. Thus the chemistries and instrumentation are intimately linked.

At present, there are three basic ways in which real-time instrumentation can supply the excitation energy for fluorophores: by lamp, light-emitting diode (LED), or laser (Fig. 5). Lamps are classified as broad-spectrum emission devices, whereas LEDs and lasers are narrow spectrum. Instruments that utilize lamps (tungsten halogen or quartz tungsten halogen) may also include filters to restrict the emitted light to specific excitation wavelengths.

Instruments using lamps include Applied Biosystem's ABI Prism 7000, Stratagene's Mx4000 and Mx3000P, and Bio-Rad's iCycler iQ. LED systems include Roche's LightCycler, Cepheid's SmartCycler, Corbett's Rotor-Gene, and MJ Research's DNA Engine Opticon 2. The ABI Prism 7900HT is the sole machine to use a laser for excitation.

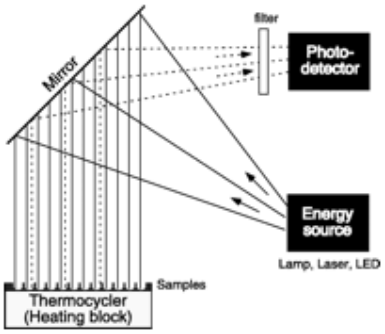

Fig. 5. Real-time PCR hardware. Samples are placed in a thermocycler, which controls the temperatures required for each cycle of PCR. The samples are exposed to excitation energy, and the resulting fluorescence is measured by a photodetector with each cycle. LED, light-emitting diode.

To collect data, the emission energies must also be detected at the appropriate wave lengths. Detectors include charge-coupled device cameras, photomultiplier tubes, or other types of photodetectors. Narrow wavelength filters or channels are generally employed to allow only the desired wavelength(s) to pass to the photodetector to be measured. Usually, multiple discrete wavelengths can be measured at once, which allows for multiplexing, i.e., running multiple assays in a single reaction tube. Another portion of the instrumentation consists of a thermocycler to carry out PCR. Of particular importance for real-time PCR is the ability of the thermocycler to maintain a consistent temperature among all sample wells, as any differences in temperature could lead to different PCR amplification efficiencies. This is accomplished by using a heating block (Peltier based or resistive), heated air, or a combination of the two. As one might expect, heating blocks generally change temperature more slowly than heated air, resulting in longer thermocycling times. For example, Roche's LightCycler models utilizing heated air can perform 40 cycles in 30 min, whereas Applied Biosystem's ABI Prism 7900HT utilizing a Peltier-based heating block takes 1 h 45 min. For further details on real-time PCR instrumentation, resources include *Real-Time PCR: an Essential Guide* (Edwards, 2004) for a side-by-side comparison or the respective manufacturer's website for the most up to date model information.

Real-time instrumentation certainly would not be complete without appropriate computer hardware and data-acquisition and analysis software. Software platforms try to simplify analysis of real-time PCR data by offering graphical output of assay results including amplification and dissociation (melting point) curves (Fig. 3). The amplification curve gives data regarding the kinetics of amplification of the target sequence, whereas the dissociation curve reveals the characteristics of the final amplified product.

ADVANTAGES AND LIMITATIONS OF REAL-TIME PCR QUANTITATION

There are many methods in molecular biology for measuring quantities of target nucleic acid sequences. However, most of these methods exhibit one or more of the following shortcomings: they are time consuming, labor intensive, insufficiently sensitive, nonquantitative, require the use of radioactivity, or have a substantial probability of cross contamination (Reischl et al. 2002). These methods include but are not limited to Northern and Southern hybridizations, HPLC, scintillation proximity assay, PCR-ELISA, RNase protection assay, in situ hybridization, and various gel electrophoresis PCR end-point systems. Real-time PCR has distinct advantages over these earlier methods for several reasons. Perhaps the most important is its ability to quantify nucleic acids over an extraordinarily wide dynamic range (at least 5 log units). This is coupled to extreme sensitivity, allowing the detection of less than five copies (perhaps only one copy in some cases) of a target sequence, making it possible to analyze small samples like clinical biopsies or miniscule lysates from laser capture microdissection. With appropriate internal standards and calculations, mean variation coefficients are 1–2%, allowing reproducible analysis of subtle gene expression changes even at low levels of expression (Klein 2002, Luu 2005). In addition, all real-time platforms are relatively quick, with some affording high-throughput automation. Finally, real-time PCR is performed in a closed reaction vessel that requires no post-PCR manipulations, thereby minimizing the chances for cross contamination in the laboratory. However, there are several limitations to real-time PCR methods. The majority of these are present in all PCR or RT-PCR-based techniques. Real-time PCR is susceptible to PCR inhibition by compounds present in certain biological samples. For example, clinical and forensic uses for real-time PCR may be affected by inhibitors found in certain body fluids such as hemoglobin or urea (Wilson 1997). Food microbiological applications may encounter organic and phenolic inhibitors (Wilson, 1997). To circumvent this problem, alternative DNA polymerases (e.g., *Tfl*, *Pwo*, *Tth*, etc.) that are resistant to particular inhibitors can be used. Other limitations primarily concern real-time PCR-based analysis of gene expression (Ausubel et, al. 2005, Ballard et, al. 2000, Barthe et, al. 2001, BioInformatics, 2003, Brand et, al. 2003, Brazeau, 2004, Brown et, al. 2001, Brown, and Botstein, 1999 and Bustin, 2000.)
). Because of the necessary use of RNA in an extra enzymatic step, more problems have the opportunity to occur. RNA itself is extremely labile compared with DNA, and therefore isolation must be carefully performed to ensure both the integrity of the RNA itself and the removal of contaminating nucleases, genomic DNA, and RT or PCR inhibitors. This can be a problem with any sample source, but clinical samples are of special concern because inconsistencies in sample size, collection, storage, and transport can lead to a variable quality of RNA templates. Conversion of RNA to cDNA during the RT reaction is also subject to variability because multiple reverse transcriptase enzymes with different characteristics exist, and different classes of oligonucleotides (e.g., random, poly-dT, or gene-specific primers) can be used to prime RT.

Probably the largest present limitation of real-time PCR, however, is not inherent in the technology but rather resides in human error: improper assay development, incorrect data analysis, or unwarranted conclusions. In our experience using real-time PCR for gene expression analysis, real-time PCR primer sets must be designed and validated by stringent criteria to ensure specificity and accuracy of the results (Fig. 3).

For microbiology, false positives or negatives must be considered when designing an assay to detect pathogens. Amplification and melting curves must be visually inspected while independent calculations based on these curves should be double-checked for accuracy. Real-time PCR gene expression analysis measures mRNA levels and, therefore, only suggests possible changes in protein levels or function rather than demonstrating them. And although there is a tight connection between gene expression and gene product function (Brown, 1999), this is certainly not always the case, and formal demonstration may be needed for a given research project. Of course, conclusions based on data derived from real-time PCR are best utilized when the biological context is well understood (Bustin, 2002).

COMMON APPLICATIONS FOR REAL-TIME PCR

Relative and absolute quantitation of gene expression

To evaluate gene expression, RNA must first be isolated from the samples to be studied. After isolation, RNA is linearly converted to cDNA, which is used for real-time PCR. Amplification curves are graphed by the software to help determine the "cycle time" at which fluorescence reaches a threshold level (C_T; Fig. 3). This C_T value is inversely proportional to the amount of specific nucleic acid sequence in the original sample. Both relative and absolute quantitation of gene expression utilizes the C_T value to quantitate cDNA and thereby determine gene expression. In a perfectly efficient PCR, the amount of amplified product doubles each cycle. Therefore, a difference of 1 between sample C_Ts means that the sample with the lower C_T value had double the target sequence of the other sample; a change in C_T of 2 means a fourfold difference; a change in C_T of 3 means an eightfold difference, and so on ($\Delta C_T = 2^{-\Delta CT}$ fold change). Relative quantitation measures changes in the steady-state levels of a gene of interest relative to an invariate control gene. Housekeeping genes (e.g., cyclophilin, glyceraldehyde-3-phosphate dehydrogenase, ribosomal protein 36B4, β-actin, 18S rRNA, transferrin receptor, etc.) (Dheda 2004) that are not expected to change under the experimental conditions serve as a convenient internal standard. Because the absolute quantity of the internal standard is not known, only relative changes can be determined by this method. This may not pose a problem for most research projects because the fold change may be informative irrespective of the absolute value. The limitations of this approach include a lack of absolute quantitation and the necessity for unchanging housekeeping genes as internal standards. Absolute quantitation attempts a more ambitious task to measure the actual nucleic acid copy number in a given sample. This requires a sample of known quantity (copy number) of the gene of interest that can be diluted to generate a standard curve. This is an external "absolute" standard. Unknown samples are compared with the standard curve for absolute quantitation (Kuhne and Oschmann 2002). The primary limitation to this approach is the necessity of obtaining an independent reliable standard for each gene to be analyzed and then running concurrent standard curves during each assay.

Validation of DNA microarray results

Because of the reliability of real-time PCR, many researchers use the above relative or absolute quantitation of gene expression to validate and corroborate the results of printed DNA microarrays or oligonucleotide arrays (e.g., Affymetrix GeneChip).

Arrays are used because they allow a researcher to look in an "unbiased" fashion at how experimental manipulation might affect any of the thousands of genes present on the array. Some arrays purport to contain the entire "genome" of a model organism and thus can theoretically be probed to comprehensively determine changes in expression within the entire "transcriptome." The problem is that there can be artifacts, and it is often difficult to get reliable quantitative data or adequate statistical power with present array technology. Thus many researchers choose real-time PCR as a supporting technique to validate and better quantitate the most interesting candidate genes from their arrays.

Counting bacterial, viral, or fungal loads

Real-time PCR can distinguish specific sequences from a complex mixture of DNA. Because of this, it is useful for determining the presence and quantity of pathogen-specific or other unique sequences within a sample.

Identification of mutations (or single nucleotide polymorphisms) by melting curve analysis

Real-time PCR is ideally suited for the analysis of mutations, including single nucleotide polymorphisms (SNPs), often replacing other techniques such as sequencing, single-strand conformation polymorphism assays, and restriction fragment-length polymorphism analysis (Edwards et al. 2004). To detect mutations in the sequence, melting curve analysis is automatically done on the amplified product (amplicon) immediately after PCR thermo cycling and fluorescence measurement. Although any of the above-described fluorescent chemistries will work for detection of mutations, hybridization probes are often used. After PCR is complete, the hybridization probes are attached to the amplicon in tandem, allowing energy to transfer from the donor to acceptor fluorophore, which emits a signal. As the temperature of the reaction vessel increases during the melting curve analysis, the donor probe will dissociate, resulting in a decrease of fluorescence. If there are any mutations in the amplicon (in the hybridization region), the donor probe will bind less strongly and dissociate at a lower temperature. Thus mutations can easily be detected by observing a shift in the melting point of the PCR product. This type of analysis can also be used for genotyping individuals or experimental organisms (i.e., allelic discrimination).

FIELDS OF REAL-TIME PCR APPLICATION

Because of the power of real-time PCR applications, it is already used in many different fields within biomedical research and molecular diagnostics.

Biomedical research

Real-time PCR has become quite commonplace in basic research within the biomedical sciences. Any time gene expression data is desired for a particular research project, real-time PCR is likely to be used. Therefore, real-time PCR has impacted a wide variety of topics of study, and the examples of gene expression analyses are innumerable. However, there are additional applications that are particularly useful to basic research. Real-time PCR can be used for genotyping knock out, knock in, and transgenic mouse models or for determining the efficacy of gene knock down and delivery methods in animals or cell culture systems.

With the use of real-time PCR's capability of allelic discrimination, detection of SNPs that may predispose individuals to particular diseases can be determined in populations, thereby facilitating epidemiological studies.

Molecular diagnostics

Clinical microbiology diagnostic laboratories can use real-time PCR to detect changes in viral load. Because viral load and disease severity are related, real-time PCR can measure the disease progression and efficacy of antiviral therapies. Mutation analyses using melting curves enable individual and epidemiological studies of viral coinfections or quasispecies (Mackay, 2002).

Real-time PCR most notably benefits patients when used to detect and identify bacteria (Mackay, 2004). Quick and early detection allows the clinician to immediately prescribe better targeted antibiotic therapies and could, in the long term; help reduce the use of broad-spectrum antibiotics, which may encourage the emergence of antibiotic-resistant strains. Real-time PCR has been used to detect *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Listeria monocytogenes*, and *Neisseria gonorrhoeae* (Ballard et al. 2000, Cleary et al. 2003, Lunge et al. 2002, Whiley et al. 2002). Mutation (melting curve) analysis has been able to monitor antibiotic resistance among *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Helicobacter pylori*, *Enterococcus faecalis*, and *Enterococcus faecium* (Gibson et al. 1999, Martineau et al. 2000, Woodford et al. 2002). This technology has been extended to quickly detect spores of *Bacillus anthracis*, the well-known causative agent of anthrax and a potential weapon of biological warfare (Makino et al. 2001).

Real-time PCR is revolutionizing microbiological diagnostics because of the sensitivity of detection and specificity for determination of variants. In addition, there may be substantial time and cost savings over traditional culture methods for determining the quantity of a given pathogen in a clinical specimen. In fact, the Mayo Clinical Microbiology Laboratory has decreased the analytic turnaround time for six different pathogens from a range of 1–14 days by traditional methods to 30–50 min using real-time PCR and has obtained these results with similar or better sensitivities (Reischl, 2002). Of course, there are limitations. Real-time PCR will measure DNA from both live and dead pathogens, whereas traditional culture methods focus on measuring live pathogens (Hein et al. 2001). Accordingly, assays for particular pathogens must be responsibly designed and strictly evaluated before conclusions can be made (Klein, 2002).

The ability to identify specific DNA sequences is also critical in clinical oncology. For example, real-time PCR can be used to detect and sometimes quantify chromosomal translocations or their fusion gene transcripts present in a patient sample for use in the determination of minimum residual disease (MRD) or disease progression. This technique has been used to detect MRD in patients by measuring the AML-1/MTG8 fusion gene product of acute myeloblastic leukemia (Krauter et al. 2001), several gene product rearrangements of acute lymphoblastic leukemia (Pongers-Willemse et al. 1998), or the patient response to interferon-α treatment by measuring the BCR-ABL fusion gene product of chronic myeloid leukemia (Barthe et al. 2001).

Real-time PCR can also be used to determine the DNA copy number that can lead to malignancy (Ginzinger et al. 2000) or be used to analyze gene expression in solid tumors using very small specimens like fine-needle aspirates (Ohnmacht et al. 2001). Such sensitive and precise detection may prove useful not just for a better understanding of cancer and tumor biology but also for determining more efficacious therapeutic strategies and better stratification of a patient's risk for recurrence of disease. Real-time PCR can be used to aid drug discovery and characterization by determining the kinetics of target gene expression in response to drugs and the response of transporters or metabolizing enzymes that facilitate distribution or disposal (Brazeau 2004).

FUTURE APPLICATIONS AND PERSPECTIVES

Any need for fast and precise measurement of small amounts of nucleic acids represents a potential future niche for real-time PCR-based innovations. As machines become faster, cheaper, smaller, and easier to use through competition, standardized assay development, and advances in microfluidics (Mitchell 2001), optics, and thermocycling, more in-field application needs are likely to be filled. In the commercial food industry and agriculture, real-time PCR will likely see expanded use for the detection and identification of microbes, parasites, or genetically modified organisms. Forensics will benefit from real-time PCR's sensitivity, specificity, and speed, especially because time is crucial to many criminal investigations and specimen size may be limited. Reduced cost and increased portability open the door for the diagnosis of diseases in remote areas along with on-site epidemiological studies and may facilitate the transfer of needed scientific technologies to developing countries, thereby contributing to their "scientific capacity" (Harris 2004). Because the demand to measure gene expression is unlikely to wane as long as biomedical science is thriving, new generations of real-time PCR machines are likely to be developed. Much like computers, earlier generations of machines should be relatively inexpensive and therefore increase global access to the technology. Hopefully, this technology will enter the classroom to enable life science educators to better equip students and encourage them to consider careers in science. Teaching real-time PCR could be used both as a platform to introduce key concepts in molecular biology as well as a chance to give students confidence by successfully learning and implementing scientifically relevant skills. This would be a great way to increase hands-on learning, which may be a key component to improving biology education in the United States (Stokstad 2001). Real-time PCR generates a focused look at the "transcriptome," enabling researchers to better understand the transcriptional programs that underlie physiology, pathophysiology, and development. Understanding gene expression narrows the gap in our knowledge between the "instructions" (the genome) and the "functions" (gene products) of biology. The evolution of the science and technology of molecular biology can be viewed chronologically as epochs hallmarked by the maturation of study of particular biomolecules. Extensive, and in some ways comprehensive, analysis of DNA (genomics) was first realized in the Human Genome Project. Now analysis of RNA expression profiles (transcriptomics) is reaching maturity. The imminent future promises great leaps forward in the analysis of proteins (proteomics), whose technology is the most rapidly growing, and in the analysis of biological lipids or metabolic intermediates (lipomics or metabolomics, respectively).

Hopefully, the pieces of the biomolecular puzzle can be put together, leading to a more holistic understanding of biology. To form a coherent picture, however, parallel advances in data acquisition, compilation, and analysis will be necessary to help deal with the enormity of data. The promise of such extensive knowledge of biological systems is staggering but will certainly require dedicated individuals in all biomedical fields to figure out how best to utilize the new technologies and the information produced by them.

AN EXAMPLE OF REAL-TIME PCR FOR RELATIVE QUANTITATION OF GENE EXPRESSION

Activation of peroxisome proliferator-activated receptor-α enhances expression of adiponectin receptors in the liver.

The superfamily of nuclear receptors includes ligand-activated transcription factors, which can respond to hormonal or metabolic ligands. Because these proteins regulate the transcription of target genes and thereby change mRNA levels, their activities are particularly amenable to study by the methodology of real-time RT-PCR.

Peroxisome proliferator-activated receptor (PPAR)-α, -β (-δ), and -γ are members of the nuclear receptor superfamily and known to be activated by endogenous saturated and unsaturated long-chain fatty acids, eicosanoids, and prostaglandins. In addition, two classes of antidiabetic agents are known to bind PPARs: fibrates (e.g., fenofibrate, known clinically as Lofibra or Tricor) bind PPAR-α, whereas thiazolidinediones (e.g., rosiglitazone, known clinically as Avandia) bind PPAR-γ. The binding of these drugs activates the respective transcription factor to enhance the transcription of target genes and affect a physiological response. Because PPAR-α activation favors fatty acid oxidation in tissues (especially the liver and heart), it may enhance insulin sensitivity by reducing intracellular fatty acid accumulation. PPAR-γ activation favors the storage of lipids in adipose tissue, thereby protecting the rest of the body from lipid overload and insulin resistance (Ferre 2004). Activation of PPARs may also enhance insulin sensitivity in other ways. For example, PPAR-γ is known to upregulate adiponectin (Brand et al. 2003, Ye et al. 2003). Adiponectin is considered to be an "adipocytokine" because it is made exclusively by adipose tissue and then secreted into the circulation. Plasma adiponectin levels appear to be inversely correlated with obesity and insulin resistance. Adiponectin action on tissues became better understood when two receptors for adiponectin were recently identified (Yamauchi et al. 2003). Adiponectin receptor 1 (AdipoR1) is expressed ubiquitously but most highly in skeletal muscle, whereas AdipoR2 is primarily expressed in the liver (Yamauchi et al. 2003). Because the adiponectin system is already implicated in enhancing insulin sensitivity, we wanted to know whether insulin sensitization by PPARs could in part be due to their potential effects on adiponectin receptor expression. In these experiments, we sought to determine the impact of various nuclear receptor agonists (drugs) and a gene knockout (genes) on the expression of adiponectin receptors in the liver (Fig. 6). First, mice were fed standard chow supplemented with 30 mg/kg LG268 [retinoid X receptor (RXR)], 0.5% (wt/wt) fenofibrate (PPAR-α), 150 mg/kg troglitazone (PPAR-γ), 0.05% (wt/wt) prenenolone-16α-carbonitrile [pregnane X receptor (PXR)], 3 mg/kg TCPOBOP (constitutive androstone receptor), 0.5% (wt/wt) chenodeoxycholic acid (farnesoid X receptor), 50 mg/kg T1317 [liver X receptor (LXR)], or 30 mg/kg LG268 + 50 mg/kg T1317 (RXR+LXR) for 12 h.

Second, both wild-type and PPAR-α knockout mice were fed a standard chow with or without 0.5% (wt/wt) fenofibrate for 7 days. Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center (Dallas, TX). Tissues were harvested, RNA was extracted, real-time PCR was performed to determine the relative abundance of mRNA (Kurrasch et al. 2004), and calculations were done using the comparative C_T method (User Bulletin No. 2, Perkin-Elmer Life Sciences). Data were procured by analyzing the expression in individual livers (triplicate measurement) in each group (n = 3–4) and are expressed as means ± SE. Thus the SE reflects biological variation in addition to measurement variation. In the first experiment, AdipoR1 mRNA levels were mildly increased by activation of RXR and PPAR-α (1.34 ± 0.15- and 1.70 ± 0.12-fold), as were AdipoR2 mRNA levels (1.70 ± 0.07- and 2.31 ± 0.24-folds). Notably, the PPAR-γ agonist had no effect. In the second experiment, AdipoR1 and AdipoR2 mRNA expression were increased 1.91 ± 0.05- and 3.28 ± 0.10-fold, respectively, in wild-type animals treated with fenofibrate. No increase was observed for either gene in treated PPAR-α knockout animal. Similar results were observed in livers of mice treated with another PPAR-α agonist, GW7647, by oral gavage [2 doses of 5 mg/kg over 14 h; data not shown (Brown et al. 2001)].

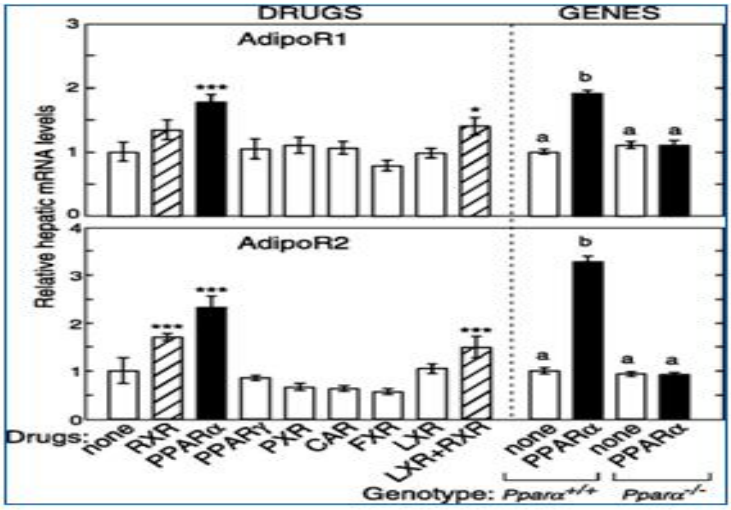

Fig. 6. Example of real-time PCR analysis.

Mice received drugs that specifically activate various nuclear hormone receptors (listed on the *x*-axis). Liver RNA was prepared, and the RNA levels for adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2) were measured using an ABI Prism 7900HT system, the SYBR green I method, and target-specific primers (AdipoR1: forward 5′-cagtgggaccggtttgc-3′ and reverse 5′-aagccaagtcccaggaacac-3′; AdipoR2: forward 5′-aacgaatggaagagtttgtttgtaa-3′ and reverse 5′-gtagcacatcgtgagggatca-3′). Full details of the method can be found (Kurrasch et al. 2004), and cyclophilin was used as the invariate housekeeping gene. *Left*: only peroxisome proliferator-activated receptor (PPAR)-α (solid bars) and its dimeric partner retinoid X receptor (RXR; hatched bars) elicit an increase in hepatic AdipoR1 and AdipoR2 mRNA levels compared with the control group (**P* < 0.05 and ****P* < 0.001).

Right: the importance of PPAR-α is highlighted, because in mice lacking this nuclear hormone receptor (Ppar-$\alpha^{-/-}$) the fibrate drug fails to work. [a,b]By ANOVA, groups sharing a common letter designation are not different. PXR, pregnane X receptor; CAR, constitutive androstane receptor; FXR, farnesoid X receptor; LXR, liver X receptor.

These results demonstrate that fenofibrate enhances adiponectin receptor expression in the liver by a PPAR-α-dependent mechanism and suggest that fenofibrate may enhance insulin sensitivity by increasing adiponectin action on the liver. Further investigation is needed to determine whether PPAR-α is acting directly on the promoters of AdipoR1 and AdipoR2 genes in liver cells or indirectly by some other means (e.g., changes in metabolism). Also, this result is consistent with the therapeutic potential of PPAR-α/γ dual agonists (Brand et al. 2003, Ye et al. 2003).

This example is a testimony to the power of real-time PCR in elucidating molecular events that underlie physiology. By applying a single methodology, that of real-time PCR, to a drug-treated mouse knockout model, we were able to gain insight into novel molecular mechanisms potentially involved in insulin sensitization. Obviously, the ability to precisely detect relative changes in gene expression is a valuable tool for studying any number of physiological, pathophysiological, and developmental models. Thus real-time PCR can be a powerful first step in many biomedical research projects and programs.

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