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Review on Principles of Real Time Polymerase Chain Reaction (PCR)

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Abstract: Real-time Polymerase chain reaction (Quantitative PCR), which allows for quantitative measurement of DNA or RNA molecules, is one among different kinds of PCR. Real-time PCR instrumentation was first made commercially available by Applied Bio-systems in 1996, after which several other companies added new machines to the market. The primary difference between real-time and conventional PCR assays is that products of a real time reaction are measured in "real time", as the PCR reaction is being performed, rather than after the reaction is complete. 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. A single molecular beacon is used for detection of a PCR amplification product and multiple beacon probes with different reporter dyes are used for single nucleotide polymorphism detection. The most commonly used methods for DNA preparation are based upon one of four systems: biphasic purification, silica-gel based column purification, magnetic bead purification and boiling with chelation of PCR inhibitors. Real-time PCR is used for absolute and relative quantifications of DNA and RNA template molecules and for genotyping in a variety of applications. As a limitation of Real time PCR, false-positive results are inevitable due to its high sensitivity, typically requiring no more than 10 copies of RNA or DNA for detection. When assays are adopted from other sources, careful attention should be paid to any variables which may not be identical; examples include changing the platform, using a different formulation of master mix, beginning with a different sample type or extraction method and changing either the template or reaction volume.

Key words: Principle · Real Time PCR · Review

INTRODUCTION

Real-time PCR is a powerful, economical, rapid and high-throughput technique for assaying gene expression with broad applications in clinical studies, diagnostics, forensics, food technology, pathogen detection and functional genomics. The technique has been adopted quite widely because of the apparent advantages over other gene expression methodologies such as Northern blot analysis: a much larger dynamic range, higher sensitivity, smaller sample amount requirements and less labor [1]. Real-time PCR instrumentation was first made commercially available by Applied Bio-systems in 1996, after which several other companies added new machines to the market. Presently, Applied Bio-systems, Bio Gene, Bioneer, BioRad, Cepheid, Corbett Research, Idaho Technology, MJ Research, Roche Applied Science and Strata gene all offer instrumentation lines for real-time PCR [2].

For the production of viral vaccines, measuring the exact titer of virus is important to make a vaccine of the correct dosage. The plaque and tissue culture infectious dose 50 (TCID50) assays are commonly used methods to quantify virus titer. However, these methods are time consuming (~1 week) [3] and not very cost effective. Compared with these traditional methods, a real-time PCR method has many advantages, including rapidity, quantifiable measurement, higher sensitivity, higher specificity and ease of standardization [4].

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The primary difference between real-time and conventional PCR assays is that products of a real time reaction are measured in "real time", as the PCR reaction is being performed, rather than after the reaction is complete. In real-time PCR, a fluorescent detector is added to the PCR and the fluorescence of each sample is measured during each cycle of amplification. The use of fluorescence to detect PCR amplicons improves the dynamic range for real-time PCR. In the earliest form of real-time PCR, ethidium bromide, a fluorescent dye which intercalates into dsDNA, was used for detection [5] several additional dsDNA dyes, with improved sensitivity and reduced toxicity, were subsequently validated and have replaced the use of ethidium bromide [6].

From a practical standpoint, real-time PCR eliminates the necessity of post-PCR processing, reducing labor, minimizing the time required to obtain a result and preventing contamination of the laboratory from amplicon handling. However, the sensitivity of real-time PCR makes these assays more sensitive to PCR inhibitors that may not be removed by the nucleic acid extraction step, requiring careful validation of extraction procedures as well as the real-time PCR assay(s). These assays are also sensitive to false positive results caused by contamination of water sources, primers, or probes, or by aerosolization of highly concentrated templates. As a result, real-time PCR should be prepared in a designated area, separate from that used for nucleic acid extraction, which is regularly cleaned with chemicals or irradiated with UV light. Cross-contamination during the process of preparing the reaction is further minimized by maintaining a "Clean to dirty" work flow: prepare the master mix first, then handle the unknown DNA specimens and, finally, add positive controls, starting with the least concentrated template and finishing with the most concentrated. There are also several chemical options available to prevent amplicon cross-contamination [7].

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 [8].

Both proper experimental design and reliable statistical analysis are the bases for the accuracy and reproducibility of results. Furthermore, without these, data interpretation and conclusions could be faulty. Proper experimental design involves the inclusion of suitable internal and external controls, sufficient replication and appropriate and efficient cDNA synthesis methods. The optimal statistical treatment for real-time RT-PCR requires precise data modeling, integration of amplification efficiency (AE) and rational outlier exclusions [9].

In brief, the advantages of real-time PCR are exploited in clinical diagnosis and the monitoring of infectious diseases and tumors. The technique is applied for the analysis of age dependent diseases, cytokine and tissue-specific expression, forensic samples, epigenetic factors like DNA methylation and for food monitoring. The field of applications is still growing rapidly, which suggests that real-time PCR will become one of the most important techniques in molecular life sciences and medicine [10]. The objective of this seminar is to review different principles on real time Polymerase Chain Reaction (PCR).

MATERIAL AND METHODS

Chemistries of Real-time PCR: Today fluorescence is exclusively used as the detection method in real-time PCR. Both sequence specific probes and non-specific labels are available as reporters. In his initial work Higuchi used the common nucleic acid stain ethidium bromide, which becomes fluorescent upon intercalating into DNA [11]. Classical intercalators, however, interfere with the polymerase reaction and asymmetric cyanine dyes such as SYBR Green I and BEBO have become more popular [12]. Asymmetric cyanines have two aromatic systems containing nitrogen, one of which is positively charged, connected by a methine bridge. These dyes have virtually no fluorescence when they are free in solution due to vibrations engaging both aromatic systems, which convert electronic excitation energy into heat that dissipates to the surrounding solvent. On the other hand the dyes become brightly fluorescent when they bind to DNA, presumably to the minor groove and rotation around the methine bond is restricted [13].

Chemical modifications and alterations of the oligonucleotide backbone are employed in some probes to improve the binding properties to the target template.

This makes it possible to use shorter probes, which is advantageous for the detection of targets with short conserved regions such as retroviruses. Light Up probes have a neutral peptide nucleic acid (PNA) backbone that binds to DNA with greater affinity than normal oligonucleotides [14].

SYBR Green I: SYBR green I bind to the minor groove of dsDNA, emitting 1,000-fold greater fluorescence than when it is free in solution [15]. Therefore, the greater the amounts of dsDNA present 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 [8].

5' Nuclease (TaqMan) Probes: 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. These are likely the most widely used fluorogenic probe format [16] 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. In addition, hydrolysis probes allow for simple identification of point mutations within the amplicon using melting curve analysis [8].

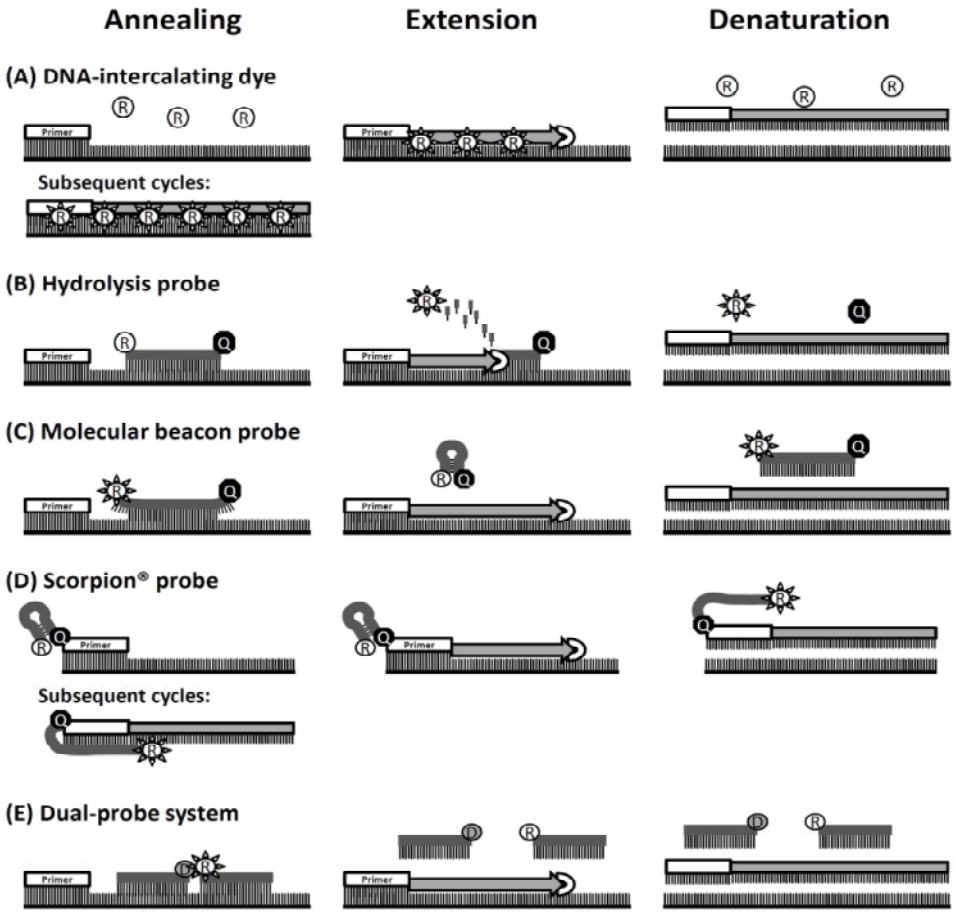
Molecular Beacons: Molecular beacons are similar to TaqMan probes but are not designed to be cleaved by the 5' nuclease activity of Taq polymerase. These probes have a fluorescent dye on the 5' end and a quencher dye on the 3' end of the oligonucleotide probe. A region at each end of the molecular beacon probe is designed to be complementary to itself, so at low temperatures, the ends anneal, creating a hairpin structure. This integral annealing property positions the two dyes in close proximity, quenching the fluorescence from the reporter dye [17].

Typically, a single molecular beacon is used for detection of a PCR amplification product and multiple beacon probes with different reporter dyes are used for single nucleotide polymorphism detection. By selection of appropriate PCR temperatures and/or extension of the probe length, molecular beacons will bind to the target PCR product when an unknown nucleotide polymorphism is present but at a slight cost of reduced specificity. There is not a specific temperature thermo cycling requirement for molecular beacons, so temperature optimization of the PCR is simplified [17].

Scorpions: Scorpions combine the detection probe with the upstream PCR primer [18] and consist of a fluorophore on the 5' end, followed by a complementary stem loop structure (Also containing the specific probe sequence), quencher dye, DNA polymerase blocker (A non-amplifiable monomer that prevents DNA polymerase extension) and finally a PCR primer on the 3' end. The probe sequence contained within the hairpin allows the scorpion to anneal to the template strand, which separates the quencher for the fluorophore and results in increased fluorescence. Because sequence-specific priming and probing is a unimolecular event, scorpions perform better than bimolecular methods under conditions of rapid cycling such as the Light Cycler [19]. The duplex scorpions still bind in a unimolecular event, but because the reporter and quenchers are on separate molecules, they yield greater signal intensity because the reporter and quencher can separate completely [20].

Choices for Detection Chemistry: The design of primers and probes for the PCR detection of cDNA templates is different from that for traditional DNA applications. DNA extracts from cells are typically double stranded and primers can be designed to amplify DNA from either strand. With the exception of dsRNA viruses, the cDNA produced during the reverse transcription from RNA viruses or mRNA will be ssDNA representing only one strand. Careful consideration is needed to avoid making primers or probes that do not anneal in the proper orientation for successful real time PCR. This is particularly true when molecular markers, such as fluorescent molecules or quenchers, must be cleaved from probes during polymerization [21].

Detection Based Upon Fluorescent DNA-Binding Dyes: Although early applications used ethidium bromide, dye-based detection is presently based upon more sensitive dyes, especially SYBR Green I, which intercalate into dsDNA and emit fluorescence (Figure 1A). For example, SYBR Green I, SYTO13 and FAM (A fluorochrome used in probe-based assays) are detected at the same wavelengths, making it possible to use these dyes in machines with the same detection parameters. With dye-based detection systems, the concentration of dye is constant between platforms and assays, requiring no extra validation of the detection component [21].



Source: Lyon [25].

Fig. 1: Diagram illustrating five types of detection systems commonly used for real-time PCR assays and their activity during thermocycling conditions used for annealing, extension and denaturation. For simplicity, only one strand of the template is represented. The reporter fluorophore (R), quencher (Q) and donor fluorophore (D) are included, as appropriate and distinctions are made between an inactivated reporter and a reporter that is fluorescing. During denaturation, note the lack of specific dye-based fluorescence and the presence of non-specific fluorescence exhibited by molecular beacon and Scorpion® probes; data collection for real-time PCR is typically performed during the annealing stage of the reaction [25].

Detection Based upon Labeled Oligonucleotide Probes: In probe-based detection systems, the fluorescent reporter is covalently bonded to an oligonucleotide probe that is designed to anneal to the template between the primers. All of these systems depend upon the transfer of fluorescent energy between two different molecules, either a reporter and a quencher or a reporter and a donor fluorophore. Oligonucleotide probe-based detection methods were initially based upon the annealing and hydrolysis, mediated by *Taq* polymerase, of probes labeled with a fluorescent reporter on one end and a fluorescence quencher on the other (Figure 1B). TaqMan® probes are one example of this type of chemistry. As the DNA polymerase extends the template from the primer, in a 5' to 3' direction, it also exhibits 3' exonuclease activity which degrades the probe and releases the fluorescence from the quencher. In these assays, the probe should be designed to anneal to the template at a temperature approximately 10°C higher than that of the primers, to ensure the probe is in place before the primers anneal and the polymerase begins extension [22].

This requirement can produce relatively long probes, especially when detecting GC-poor templates. If the probes are longer than 30-35 bases, quenching may be poor, resulting in high background fluorescence.

Minor-groove binding, or MGB, probes were developed to produce shorter probes with the necessary high annealing temperature for hydrolysis assays. In these probes, a moiety that binds to the minor groove of dsDNA is covalently bonded to the 3' end of the probe, in addition to the quencher, providing increased stability of the probe-template duplex and increasing the annealing temperature. LNA probes use modified nucleic acids, "Locked nucleic acids", to achieve similar results [23].

In contrast to the hydrolysis probes discussed above, molecular beacon, Scorpion® and Light Cycler (HybProbe) probes depend upon hybridization without hydrolysis. Molecular beacon probes have a stem-loop structure, with a central region that is complementary to the target DNA, flanked on both sides by short, GC rich sequences that are complementary to each other (Figure 1C). Ideally, both the central region of the probe and the GC-rich arms will anneal to their respective targets at a temperature approximately 7-10°C higher than the PCR primers but lower than the temperature used for the extension phase of the PCR. Similar to hydrolysis probes, molecular beacons are labeled with a fluorescent reporter at the 5' end and a quencher at the 3' end; the hairpin structure of the probe keeps the reporter and quencher in close physical proximity. In addition to the dual-labeled molecular beacons that couple a fluorescent reporter with a quencher, there is some chemistry available that use a single fluorophore whose emission is altered by the process of hybridization [24].

Scorpion® probes are essentially a modification of molecular beacons, in which an oligonucleotide probe, with the stem-loop structure, a reporter and a quencher, is covalently attached to the 5' end of one of the PCR primers (Figure 1D). In these systems, the primer is incorporated into the product during amplification; the probe remains attached and during subsequent cycles, the probe can hybridize to the adjacent end of the product during the annealing step and generate fluorescent signal. As with molecular beacons, the secondary structure of the Scorpion® probe-primer combination is critical. However, use of these probes reduces the number of components that must be optimized to develop the final assay [24].

Dual-probe hybridization systems (HybProbe, Light Cycler®) consists of two separate probes, or a probe and a primer, which anneal on the same strand of the template [25] (Figure 1E). The first probe is labeled with a donor fluorophore at the 3' end and the second oligonucleotide is labeled with an acceptor fluorophore at the 5' end. When the probes are both annealed to the template strand, the fluorophores are separated by a gap of 1-4 nucleotides, allowing fluorescent energy to be transferred from the donor to the acceptor. The use of two probes increases the number of nucleotides used for detection and, thus, the specificity of the assay. As with other hybridization probes, melt curve analysis can be used to assess the strength of probe annealing, with reduced temperature reported in the case of polymorphisms in either probe.

Preparation of Template: The primary concerns for nucleic acid template quality for real-time PCR are similar to those for other applications: yield, purity and lack of inhibitors. The primary difference is in the increased emphasis on consistency of yield between samples and in the removal of all PCR inhibitors: quantitative studies require that the efficiency of extraction is similar for all samples and controls included in the study and the improved sensitivity of real-time PCR for detection of template is accompanied by an increased sensitivity to PCR inhibitors. Inhibitors of PCR can co-purify with the nucleic acids, may fail to be removed by insufficient washing, or can be introduced from the laboratory environment; some common inhibitors of real-time PCR include: heme, heparin, EDTA, ethanol and compounds found in feces [21].

Methods for Preparation of DNA Templates: In general, the effects of DNase activity during sample collection can be minimized by keeping cellular membranes intact. DNases are used by cells, in a highly regulated and restricted fashion, to degrade DNA as part of normal cellular metabolism. Eukaryotic cells may have DNase in nuclear granules or lysosomes and bacteria use methylation and other strategies to protect their cytoplasmic genome from restriction enzymes and other cytoplasmic nucleases. Depending upon the type of sample and duration of storage, samples may be refrigerated (Short-term), frozen, stored in >70% ethanol or isopropanol, or dried completely on filter paper. Freeze-thaw, mechanical disruption, or chemical disruption of cellular membranes will permit the endogenous DNases to contact genomic DNA. The most commonly used methods for DNA preparation are based upon one of four systems: biphasic purification, silica-gel based column purification, magnetic bead purification and boiling with chelation of PCR inhibitors. Different methods may be better suited for different applications; published papers are available that compare DNA extraction methods for common sample types [26].

Preparation of Templates from RNA: Because RNA is rapidly degraded by free ribonucleases (RNases) in biological samples, proper collection and preservation of samples is the first step in successful reverse transcription PCR. Cells use RNA to manage most biochemical reactions and cellular regulatory activities; as a result, they must be able to rapidly degrade RNA and all cells contain RNases [27].

Viral agents with RNA genomes are different from cellular pathogens. The biochemical structure of the virion, the type of nucleic acid in the genome and the structure of those nucleic acids, varies between viral families. DNA viruses have a DNA-based genome and can be treated just like cellular pathogens for DNA extraction. RNA viruses can have single or double stranded RNA, the genome can be continuous or segmented and the single stranded RNA genomes can be in the sense or nonsense orientation. Some basic background knowledge of the viral agent being detected is important [28].

Reverse Transcription of RNA: The RNA genomes of the various families of viruses are poorly conserved between groups [29]. As a result, there are no universal reverse transcription real time PCR assays for viruses. While almost all cellular life shares numerous highly conserved genes in their genomes, such as rRNA genes, there are no conserved genes between the dozens of viral families. When diagnosing a true unknown virus, real-time PCR is probably not a good initial option.

Types of Real-Time Quantification

Absolute Quantification: Absolute quantification uses serially diluted standards of known concentrations to generate a standard curve. The standard curve produces a linear relationship between Ct and initial amounts of total RNA or cDNA, allowing the determination of the concentration of unknowns based on their Ct values [30]. This method assumes all standards and samples have approximately equal amplification efficiencies [31]. In addition, the concentration of serial dilutions should encompass the levels in the experimental samples and stay within the range of accurately quantifiable and detectable levels specific for both the real-time PCR machine and assay.

Relative Quantification: During relative quantification, changes in sample gene expression are measured based on either an external standard or a reference sample, also known as a calibrator [32]. When using a calibrator, the results are expressed as a target/reference ratio.

Applications: Real-time PCR is used for absolute and relative quantifications of DNA and RNA template molecules and for genotyping in a variety of applications [10].

Single-Target Assays: Real-time PCR has been especially useful for the detection of organisms that are difficult to cultivate, including not-yet-cultivated organisms, viruses and rickettsial agents [33] PCR, including real-time PCR, is also used for the detection and identification of cultivatable but slow-growing pathogens, including *Mycobacterium*, *Histoplasma* and *Brucella*. Likewise reverse transcriptase real time PCR can detect non-lytic viruses in cell cultures.

Multiplex Assays: Multiplexed assays offer distinct advantages when testing for a panel of pathogens, reducing both the number of reactions and the time and labor required completing testing. When multiplexed reactions replace several conventional or nested PCR, they can also be very cost-effective for screening for panels of pathogens. The transition to multiplexed reactions in a diagnostic virology laboratory, as well as some of the criteria for validation in this setting, was described by Gunson *et al*. [34].

Quantitating Gene Copies: Quantitative data have extensive applications in research. These data can be used for experiments that follow the kinetics of experimental or natural infection, to detect changes in the expression of virulence genes, to confirm changes detected using microarrays and to measure quantitative differences in cytokine mRNA production between individuals or experimental treatment groups [21]. Quantification of gene copies is most commonly achieved by assaying unknown samples at the same time as a standard curve of known, quantified positive control template. Five- or ten-fold dilution series are used to generate the series of standards to be tested; the number of copies in each standard is log-transformed and plotted against the CT and then linear regression is used to establish a line of best fit for the standard curve. The quantity of starting copies in each unknown sample is then extrapolated from this equation, based upon the CT for the sample. The standard caveats for quantitative assays apply: quantification is valid only within the dynamic range of the assay and unknown samples must fall within the upper and lower bounds of the standards which were run in the assay. As the field has advanced, several different formulas have been proposed for analyzing quantitative data, with differing assumptions in regards to reaction efficiency [35].

Advantages of Real-time PCR Quantification: 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, non-quantitative, require the use of radioactivity, or have a substantial probability of cross contamination. 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 [8]. 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 [8].

Limitations of Real-time PCR Quantification: 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. Food microbiological applications may encounter organic and phenolic inhibitors [36].

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 [8]. 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. For microbiology, false positives or negatives must be considered when designing an assay to detect pathogens.

CONCLUSION AND RECOMMENDATIONS

Although real-time PCR has been widely used in the detection of many viruses and other pathogenic microorganisms, it has limitations. For example, a matching primer pair and probe as well as special, expensive equipment are needed to run real-time PCR. Overall, the advantages offered by real-time PCR, including sensitivity, specificity, reduction in sample cross-contamination events and high-throughput capability, make this technology suitable for use in diagnostic laboratory settings. The field of applications is growing rapidly, which suggests that real-time PCR will become one of the most important techniques in molecular life sciences and medicine.

So the users are recommended:

- ✓ To circumvent this problem, alternative DNA polymerases that are resistant to particular inhibitors can be used.
- ✓ When assays are adopted from other sources, careful attention should be paid to any variables which may not be identical; examples include changing the platform, using a different formulation of master mix, beginning with a different sample type or extraction method and changing either the template or reaction volume.
- ✓ Careful review of the literature can also help to identify problems and solutions that have been reported by other laboratories.

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