RESEARCH NOTE

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Real Time PCR – a robust molecular technique for ever

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INTRODUCTION

In recent years, 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, clinical conditions, 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. 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 (Walker, 2002). Present day real-time methods generally involve fluorogenic probes that "light up" to show the amount of DNA present at each cycle of PCR. "Quantitative PCR" refers to the ability to quantify the starting amount of a specific sequence of DNA. Consequently real-time PCR is quickly becoming the method of choice to quantify nucleic acids. Real-time PCR instrumentation was first made commercially available by company called 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.

The 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.

Fig.1 Real-time PCR technology (like normal PCR) uses DNA or cDNA as a template for amplification and is highly sensitive. Because the reaction is able to efficiently amplify DNA only up to a certain quantity before the plateau effect, there by measures product formation during "exponential phase" of PCR cycles (Fig.1). Real-time RT (Reverse Trancriptase)-PCR and has become the most popular method of quantitating steady-state mRNA levels (Bustin, 2000)). The precision and sensitivity of real-time RT-PCR, is such an extent that, 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.

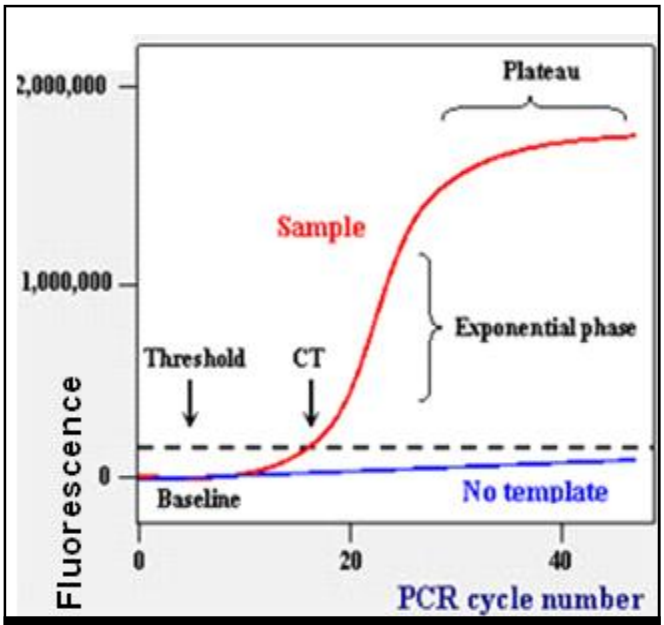

Fig. 1 : Real time PCR technology

MATERIALS AND METHODS

Real-time PCR technology consists of special fluorescent dyes\ probes in the PCR. DNA-binding dyes like EtBr or SYBR greenI, hydrolysis probes (5'-nuclease probes), and 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. 2 SYBR green I fluorescences (absorbing light of 480-nm wavelength and emitting light of 520-nm wavelength) when associated with dsDNA. 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

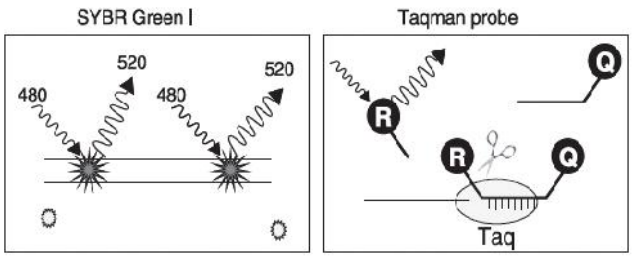

Fig. 2 : SYBR green I and Taqman probe

SYBR green I bind to the minor groove of dsDNA, emitting 1,000-fold greater fluorescence than when it is free in solution (Fig. 2) (Wittwer *et al*., 1997). 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. Other dsDNA-specific dyes (e.g., BEBO, YOYO-1, TOTO-1, etc.) have also been described but are not as widely used.

Hydrolysis probes (also called 5'-nuclease probes because the 5'-exonuclease activity of DNA polymerase cleaves the probe) offer an alternative approach to the specificity (Fig.2). These are most widely used fluorogenic probes (Mackay, 2004) are TaqMan probes. Fluorescence Resonance Energy Transfer (also called Forster transfer) in which energy is transferred from a "donor" (the reporter) to an "acceptor" (the quencher) fluorophore. During amplification, 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).

The other Fluorescence Resonance Energy Transfer based variations (Reporter-Quencher theme) includes 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 (Fig. 3).

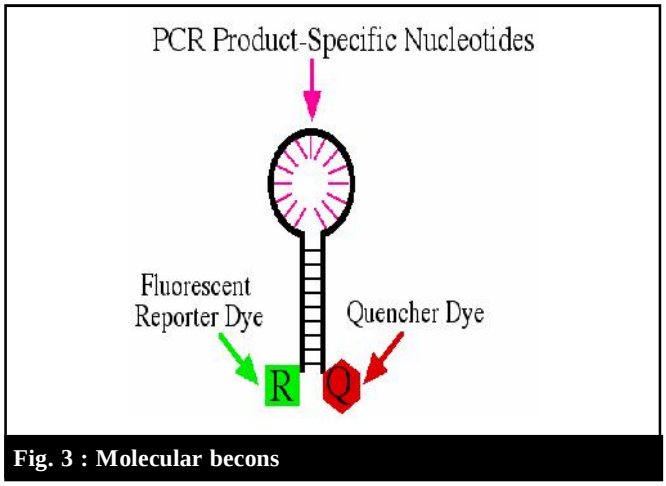

Fig. 3 : Molecular becons

Hybridization probes, uses donor and acceptor fluorophores, whereas PNAs containing thiazole orange fluorophores (called light-up probes) emit greater signal upon binding of DNA (Svanvik *et al*., 2000).

RESULTS AND DISCUSSION

The results obtained from the present investigation are summarized below :

Advantages and limitations of real-time PCR :

Advantages :

- Ability to quantify nucleic acids over an extraordinarily wide dynamic range (at least 5 log units).

- 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. (Luu *et al*., 2005)

- Real-time PCR is performed in a closed reaction condition that requires no post-PCR manipulations, thereby minimizing the chances for cross contamination in the laboratory.

Limitations :

- Real-time PCR is susceptible to PCR inhibition by compounds present in certain biological samples. For example, PCR inhibitors in certain body fluids such as hemoglobin or urea phenolic inhibitors etc.

- Real-time PCR is not inherent in the technology but rather resides in human error: improper assay development, incorrect data analysis, or unwarranted conclusions.

Applications of real-time PCR :

- Relative and absolute quantitation of gene expression: To evaluate gene expression, total RNA must be isolated from the samples to be studied. RNA is linearly converted to cDNA, which is used for real-time PCR. Amplification curves are graphed the cycle at which fluorescence reaches a threshold level (CT- cycle threshold). Both relative and absolute quantitation of gene expression utilizes the CT value to quantitate cDNA and thereby determine gene expression. Difference of 1 between sample CTs means that the sample with the lower CT value had double the target sequence of the other sample; a change in CT of 2 means a fourfold difference;and so on ($\Delta CT = 2^{-\Delta CT}$ fold change).

- Counting bacterial, viral, or fungal loads: it 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.

- Validation of DNA microarray results

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

- Biomedical research

- Molecular diagnostics.

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