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Real Time PCR: Principles and Application

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The polymerase chain reaction (PCR) has been used as the new golden standard for detecting a wide variety of templates across a range of scientific specialties and also as an essential tool in research laboratories. PCR has completely revolutionized the detection of RNA and DNA viruses[1].

Real Time vs. Traditional PCR

Real time chemistry allows the detection of PCR amplification during the early phase of the reaction. Measuring the kinetic of the reaction in the early phase of PCR provides a distinct advantage over traditional PCR detection. Traditional methods use agarose gel electrophoresis for detection of PCR amplification at the final phase or end point. End point detection is really time consuming; it takes several hours to have the result. On the other hand, results are based on size discrimination. Also, the result of end point is variable from sample to sample. While gels may not resolve this variability in yield, real time PCR is sensitive enough to detect this change.

Some problems with end point detection are: poor precision, low sensitivity, short dynamic range (<2 log), low resolution, non-automated procedure, size-based discrimination only, and post PCR processing (carry-over contamination) and results are not expressed as numbers[2].

Detection of PCR Products in Real-time

Real-time PCR and RT-PCR allow accurate quantification of starting amounts of DNA, cDNA, and RNA targets. Fluorescence is measured during each cycle, which greatly increases the dynamic range of the reaction since the amount of fluorescence is proportional to the amount of PCR product. PCR products can be detected using either fluorescent dyes that bind to double-stranded DNA or fluorescently labeled sequence-specific probes[3].

SYBR Green I

SYBR® Green I binds all double-stranded DNA molecules, emitting a fluorescent signal of a defined wavelength on binding. The excitation and emission maxima of SYBR Green I are at 494 nm and 521 nm, respectively, and are compatible for use with any real-time cycler. Detection takes place at the extension step of real-time PCR. Signal intensity increases with increasing cycle number due to the accumulation of PCR product. Use of fluorescent dyes enables analysis of many different targets without having to synthesize target-specific labeled probes. However, nonspecific PCR products and primer-dimers will also contribute to the fluorescent signal. Therefore, high PCR specificity is required when using SYBR Green I[4, 5].

Fluorescently labeled sequence-specific probes

Fluorescently labeled probes provide a highly sensitive and specific method of detection as only the desired PCR product is detected. However, PCR specificity is also important when using sequence-specific probes. Amplification artifacts such as nonspecific PCR products and primer-dimers may also be produced, which can result in reduced yields of the desired PCR product. Competition between

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the specific product and reaction artifacts can compromise assay sensitivity and efficiency. The following section discusses different formats of probe chemistry[6, 7].

TaqMan probes

TaqMan® probes are sequence-specific oligonucleotide probes carrying a fluorophore and a quencher dye. The fluorophore is attached at the 5' end of the probe and the quencher dye is located at the 3' end. During the combined annealing/ extension phase of PCR, the probe is cleaved by the 5' 3' exonuclease activity of Taq DNA polymerase, separating the fluorophore and the quencher dyes. This results in detectable fluorescence that is proportional to the amount of accumulated dyes[7, 8].

FRET probes

PCR with fluorescence resonance energy transfer (FRET) probes such as LightCycler® hybridization probes use two labeled oligonucleotide probes that bind to the PCR product in a head-to-tail fashion. When the two probes bind, their fluorophores come into close proximity, allowing energy transfer from a donor to an acceptor fluorophore. Therefore, fluorescence is detected during the annealing phase of PCR and is proportional to the amount of PCR product. FRET probes usually carry dyes that are only compatible for use on the LightCycler® machine. As the FRET system uses two primers and two probes, good design of the primers and probes is critical for successful results[9, 10].

Other system: Recently, other systems such as Molecular Beacons, Scorpion or Luxprimer are also available in market and their description is beyond the scope of this review.

Methods in Real-time PCR

Two-step and one-step RT-PCR

cDNA synthesis uses reverse transcriptases, which are enzymes generally derived from RNA-containing retroviruses. RT-PCR can take place in a two-step or one-step reaction. With two-step RT-PCR, the RNA is first reverse-transcribed into cDNA using oligo-dT primers, random oligomers, or gene-specific primers. An aliquot of the reverse-transcription reaction is then used for analysis of gene expression levels or viral load. RNA first needs to be transcribed and subsequently added to the real-time PCR. In two-step RT-PCR, it is possible to choose between different types of RT primers, depending on experimental needs. The use of oligo-dT primers or random oligomers for reverse transcription means that several different transcripts can be analyzed by PCR from one RT reaction. In addition, precious RNA samples can be immediately transcribed into more stable cDNA for later use and long-term storage. In one-step RT-PCR -also referred to as one-tube RT-PCR- both reverse transcription and amplification take place in the same tube, with reverse transcription preceding PCR. This is possible due to specialized reaction chemistries and cycling protocols. The fast procedure enables rapid processing of multiple samples and is easy to automate. The reduced number of handling steps results in high reproducibility from sample to sample and minimizes the risk of contamination since less manipulation is required. The advantages of each method are given below[11, 12].

DNA as Template in Real-time PCR

In contrast with RNA, which requires conversion into cDNA, purified genomic DNA or plasmid DNA can be directly used as starting template in real-time PCR. Not only can genomic DNA be quantified in real-time PCR, for example, in detection of bacterial DNA, but it can also be used for qualitative analysis, such as single nucleotide polymorphism (SNP) detection. SNP analysis involves the detection of single nucleotide changes using two probes labeled with different fluorophores. One probe is specific for the wild-type allele, the other for the mutant allele. Real-time PCR is highly suited for the detection of small sequence differences, such as SNPs and viral variants[13, 14, 15].

Quantification of Target Amounts

With PCR, minute amounts of starting template DNA or cDNA can be amplified enabling detection of a target sequence. If PCR products are analyzed by endpoint analysis, quantification is not possible as most reactions will already have reached the plateau phase of amplification. During this phase, no significant increase in PCR product amount takes place. This is mainly due to depletion of PCR components and renaturation of PCR product strands caused by the high concentration of end products, which prevents further primer annealing. If identical template amounts are used, this may not necessarily result in identical yields of PCR products. Equally, if different amounts of template are used, the yield of PCR products may be similar making quantification impossible. Real-time PCR

overcomes this problem by determining the actual amount of PCR product present at a given cycle, indicated by the intensity of fluorescence[16, 17].

Quantification

Target nucleic acids can be quantified using either absolute quantification or relative quantification. Absolute quantification determines the absolute amount of target (expressed as copy number or concentration), whereas relative quantification determines the ratio between the amount of target and an endogenous reference molecule, usually a suitable housekeeping gene. This normalized value can then be used to compare, for example, differential gene expression in different samples.

Absolute Quantification

Use of external standards enables the expression level of a gene to be given as an absolute copy number. For gene expression analysis, the most accurate standards are RNA molecules of known copy number or concentration. Depending on the sequence and structure of the target and the efficiency of reverse transcription, only a proportion of the target RNA in the RNA sample will be reverse transcribed. The cDNA generated during reverse transcription then serves as template in the subsequent real-time PCR. The use of RNA standards takes into account the variable efficiency of reverse transcription as well as the possible presence of reverse-transcription inhibitors, which may be in the RNA sample[18, 19].

Relative Quantification

Alternatively, gene expression levels can be calculated by determining the ratio between the amount of a target gene and an endogenous reference gene that is in all samples. This ratio is then compared between different samples. Usually, housekeeping or maintenance genes are chosen as an endogenous reference. The target and the reference genes are amplified from the same sample, either separately or in the same reaction (duplex real-time PCR). The normalized value is determined for each sample and can be used to compare, for example, differential expression of a gene in different tissues. However, the expression level of the endogenous reference gene must not vary under different experimental conditions or in different states of the tissue (e.g., "stimulated" versus "unstimulated" samples). When gene expression levels are compared between samples, the expression level of the target is referred to as being, for example, 100-fold higher in stimulated cells than in unstimulated cells. The quantification procedure differs depending on whether the target and the endogenous reference genes are amplified with comparable or different efficiencies[20, 21].

Viral Quantification

The possibility to detect viral DNA or RNA in a quantitative manner has already contributed significantly to the management and diagnosis of viral infections, as well as to the understanding of virus-host interactions. New developments in amplification techniques based on real-time detection, as well as automation of the whole process, will soon be introduced in a diagnostic laboratory setting, thereby enabling a rapid turnaround time to generate both quantitative and qualitative results. The development of molecular techniques that access viral load and the development of genotypic resistance have revolutionized the treatment of HIV disease. Commercially available viral load assays use a number of different approaches from reverse transcriptase PCR to branched DNA signal amplification. The drawbacks of the assay are that there is no international standard that allows comparison of viral load between assays and that the diversity of different clades of HIV results in under or the nondetection of some patients' samples. New real-time PCR assays are under development, including LightCycler- and TaqMan-based tests. The development of sequence-based genotyping assays for the detection of mutations associated with the development of the resistance to the 17 licensed drugs targeted against the pol gene of HIV have added to the improvements in patient management. However, next-generation assays must extend detection to include the gp41 fusion region and the integrase region of the genome as compounds directed against these targets move from clinical trails into licensed drugs. Also, genotypic assays must improve detection of minor species and detection of sequences from patients with low viral load number. Real-time sequence-based diagnostics remains a realistic target within the next 5 years[22, 23]. Real-time nucleic acid sequence-based amplification (NASBA) is an isothermal method specifically designed for amplification of RNA. Fluorescent molecular beacon probes enable real-time monitoring of the amplification process. Successful identification, utilizing the real-time NASBA technology, was performed on a microchip with oligonucleotides at a concentration of 1.0 and 0.1

microM, in 10- and 50-nL reaction chambers, respectively. The microchip was developed in a silicon-glass structure. An instrument providing thermal control and an optical detection system was built for amplification readout. Experimental results demonstrate distinct amplification processes. Miniaturized real-time NASBA in microchips makes high-throughput diagnostics of bacteria, viruses, and cancer markers possible, at reduced cost and without contamination[24, 25].

Instrumentation

Several instruments are available for detection of the real time PCR fluorescence. The first available, and currently the most popular, machines are the ABI 7700 (Applied biosystem) and the Roche Light® cycler. However, other systems have since become (and will become) available. Presented here, is a comparison of the above mentioned system with the Bio-Rad IQ® detection system and the Cephid Smart Cycler®.

Comparison of the Different Systems

Essentially, each real time PCR instrument consists of a computer-controlled thermocycler integrated with fluorescent detection system and dedicated software to analyze the result. Some systems can detect four different wave lengths (I-cycler,Mx4000 [stratagene] and Smart Cycler®, Version 2.0 Light Cycler®) whereas others can detect two different wavelengths(Light Cycler®). The Light Cycler® and Smart Cycler® are capable of performing rapid-cycle real time PCR because the reaction is set-up in capillaries or especially designated tubes. Both have optimized heating-cooling characteristic. A complete amplification protocol can be performed in 30-45 minutes.

The Smart Cycler® is a combination of 16 individual, one tube real time PCR units. It is capable of performing a different PCR program on each of 16 reaction tubes. This is very useful for a rapid optimization of the assay as many variables can be tested at the same time. The Bio-Rad I-cycler IQ® instrument can perform real time amplification with a temperature gradient for specific PCR steps, allowing the optimization of real time PCR assay. The spectrofluorometers in the thermal cycler have a number of differences. Laser-based systems are tuned to excite each fluorophore at a specific wavelength and provide maximum efficiency. Lamp-based systems provide a broad excitation range that can be filtered to work with a number of fluorophores. The laser source not only gives brighter illumination to the fluorophore signal, but also produces less background noise.

In conclusion, real time PCR is a powerful advancement of the basic PCR technique. The important steps in deciding which particular assay format to use are related to the type of data required. The requirement for a research laboratory is quite distinct from those of a diagnostic laboratory. For the latter, probe confirmation of the PCR product is an essential part of the assay, whereas SYBR green detection may be sufficient for many other applications such as quantifying expression of a gene. All of the real-time PCR machines analyzed are capable of detecting PCR product in real time and a specific assay can be made optionally on every system. However, there are some decisions to be made when selecting among different formats. The choice of system is dependent on individual laboratory needs.

Considering diagnostic applications, the Light Cycler® or Smart Cycler® may obtain faster results for urgent assays. This could reduce the time of analysis to result from 3-4 hours to 1.5 hours. On the other hand, if sensitivity is the most important issue, these machines, with their smaller reaction volume and consequently lower sensitivity, wouldn't be the first choice. The ABI 7700 and Bio-Rad -I-Cycler IQ® have a 96 well format, enabling higher throughput than other systems. The 384-well plates, as designed by ABI for use in the 7900 HT system, can further enhance through put.

For diagnostic application, internal control of nucleic acid isolation and PCR inhibition, it is essential to obtain valid results. This can be achieved using the system that enables multi-color detection, such as the I-Cycler IQ® and the Smart Cycler®. Recently, a multi-color format of the Light Cycler® is also present in market. Multiplex real-time PCRs can be developed for three different targets and an internal control by using the four detection wavelengths possible in multicolor detection.

As a matter of fact, the choice of which real time system to use depends on the range of application required. To achieve meaningful results, each assay must be validated and optimized for the particular system chosen[6, 7].

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