Real-Time Polymerase Chain Reaction

A Revolution in Diagnostics

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Real Time PCR is a new advancement in the field of molecular diagnostics. The evolution of capabilities of Real Time PCR from traditional PCR has provided researchers an upper hand over the quantification and detection of specific DNA sequences. During the recent years, the areas of application of Real Time PCR have expanded exponentially.

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Roughly three out of ten newborns of HIV-positive mothers are infected with the virus. Hence, it is important to identify HIV-positive babies early for appropriate treatment. But how can we recognise the infected babies? The most common test for HIV infection has been the ELISA, short for Enzyme-Linked Immuno-Sorbent Assay. In this test, blood is drawn from the patient and further processed to detect the antibodies[1] against HIV. ELISA is an effective diagnostic method that can detect HIV infections with 99% accuracy. Although ELISA works well for adults, it is not very effective in determining whether a newborn is infected or not. The reason is that every child is born with antibodies from its mother and if the mother is HIV-positive, the child's blood too will be positive for HIV antibodies. These maternal antibodies can linger in the child's blood for more than a year and hence a definitive ELISA can be performed only after ~18 months. But, children with HIV infection are generally more susceptible to infections making early treatment very important.

If we cannot determine which newborns are infected, how do we treat them? This is the dilemma physicians faced before the advent of the technique called Real-time Polymerase Chain Reaction [1]. Real-time PCR (also known as quantitative PCR or Q-PCR) specifically detects the amount of HIV virus itself, and not the antibodies made against it, by detecting the viral DNA present

[1]Antibodies are defensive-proteins made by our immune system in response to infections.

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in the sample. Using real time PCR, HIV infection, or its absence, can be detected within six weeks of birth, making it easier to decide the future course of action. Real-time PCR has turned out to be the most powerful tool for the quantitative analysis of nucleic acids for basic research and medical diagnostics.

Polymerase Chain Reaction

Studying specific gene sequences is very important for both basic and applied research like medical diagnostics. However, getting *sufficient* amount of a specific DNA fragment for the study is often a limiting factor. Polymerase Chain Reaction (PCR) is a technique that allows researchers to *amplify* a specific DNA fragment from a very small amount of starting material. Kary Mullis was awarded the 1993 Nobel Prize in Chemistry for discovering this technique. The ability to specifically amplify very minute amounts of DNA brought about a revolution not only in molecular biology but also in medical and forensic sciences. Recombinant DNA technology is dependent on the researcher's ability to manipulate DNA easily and PCR has been the major technique that has made it possible. Great achievements like cloning of mammals and the Human Genome Project would not have been possible without PCR.

The different steps involved in a typical PCR reaction are shown schematically in *Figure* 1. In the first step, a double-stranded DNA (called template) is converted to single strands using high temperature (a melting process called denaturation). Temperature is then lowered to allow complementary short oligonucleotides (called primers) to bind to these single-stranded DNA. The enzyme, a heat resistant DNA polymerase[2] in the mixture, then extends these primers using the template to synthesize a new strand. Heat resistance is very important, since at the end of each round of DNA copying, the double-stranded DNA must be melted again at high temperatures (~95 ^{o}C) in the reaction tube. So, at the end of first cycle, the number of DNA molecules is doubled. In the second cycle, the DNA is again melted to create single-stranded DNA and the cycle continues until reaction mixture is

[2] The most commonly used DNA polymerase is Taq DNA polymerase isolated from the bacterium called *Thermus aquaticus* that grows at high temperatures [2]. Taq DNA polymerase is not only efficient in polymerisation, it is also resistant to high temperatures. Most of the enzymes including DNA polymerases from normal organisms are inactivated by heat. But high temperature does not inactivate the heat-resistant Taq polymerase, making it ideal for use in PCR.

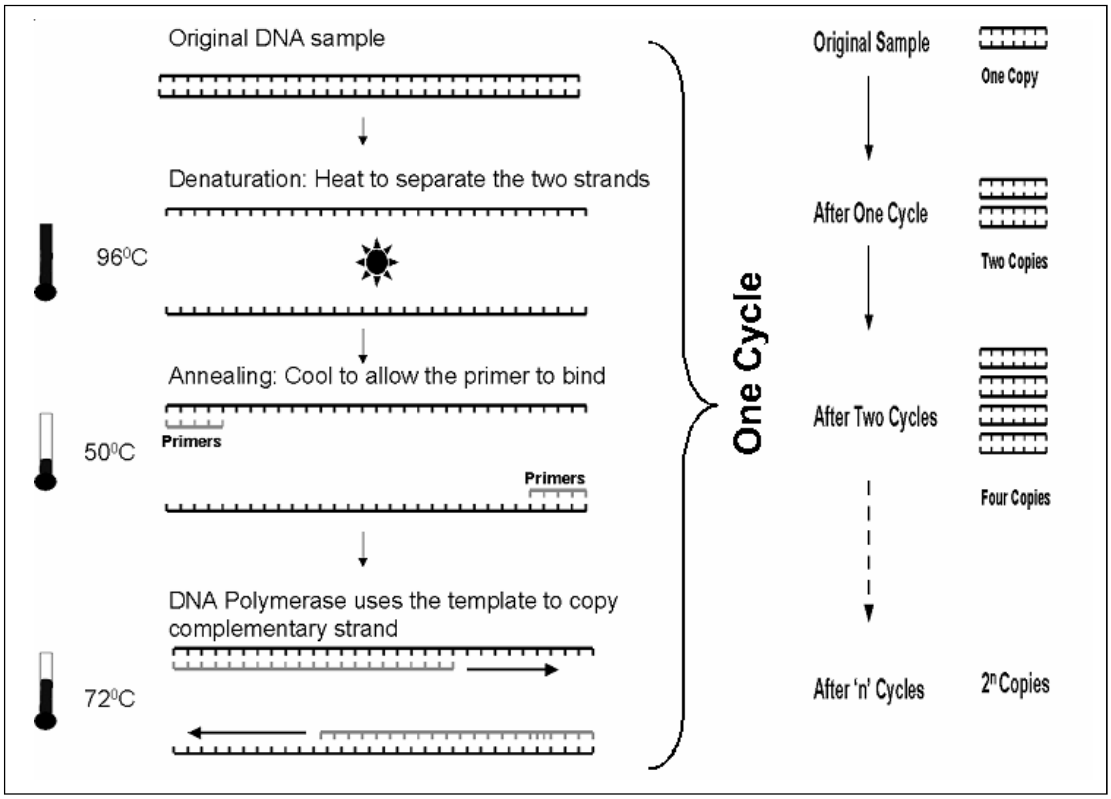

Figure 1. Schematic representation of polymerase chain reaction.

exhausted. This results in the exponential increase in the copies of the template DNA (*Figure* 1).

In traditional PCR, the product is analyzed only at the end of the reaction. The most commonly used method is agarose gel electrophoresis that separates DNA fragments on the basis of their molecular mass. At the end of the separation, the gel is stained with a dye called ethidium bromide that binds to DNA and can be visualized under UV light (left panel in *Figure* 2).

PCR is used not only to amplify DNA, but also to quantify it. As the reaction proceeds, the DNA concentration increases exponentially and plateaus off when substrates run out (*Figure* 3). If the quantification is done at the saturation stage of the PCR reaction (plateau in *Figure* 3), it is not possible to quantify the number of template DNA molecules in the original sample. As shown in *Figure* 3, the amounts of final products are same in both reac-

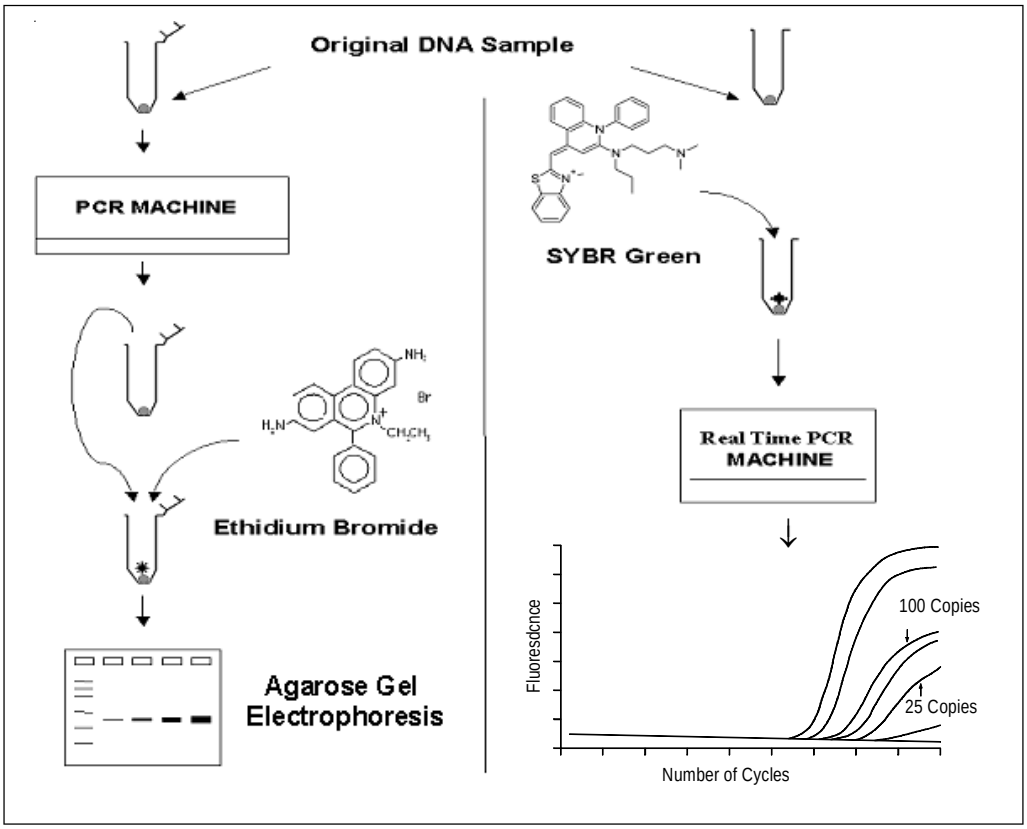

Figure 2. Differences between traditional and real-time PCR.

tions, although one had double the number of template molecules to begin with. To determine the difference in initial samples, one has to quantify the amplified products at the exponential phase (*Figure* 3), when the reaction in still going on (i.e., in *real time*).

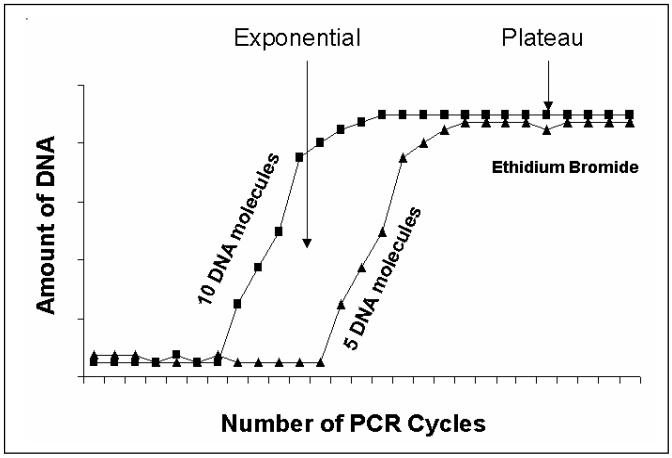

Figure 3. Different phases of polymerase chain reaction.

This, however, cannot be done by staining with ethidium bromide since its sensitivity is quite low. Due to these limitations, the amount of initial DNA in the sample cannot be quantified using traditional PCR. But if a sensitive dye binds to DNA during the exponential phase, then the difference between the fluorescence of the two samples could be used to determine their initial concentration. This technique where the amount of amplified DNA is monitored during, and not after the PCR reaction is called 'Kinetic' or 'Real-Time' PCR.

Real-time PCR

Real-time PCR is an extension of the capabilities of traditional PCR. This technique is used to amplify and at the same time quantify a specific region of a DNA molecule. As mentioned earlier, PCR amplifies specific regions of template DNA exponentially, i.e., if there is ~1 nanogram (10^{-9} g) of template DNA, by the time resources in the PCR reaction get exhausted, it is amplified up to 1 microgram (10^{-6} g). But if the initial template is in picogram (10^{-12} g) or femtogram (10^{-15} g) quantity, the amplification product would be too little to be detected using electrophoresis. This problem of sensitivity was addressed in Real-time PCR by making use of the chemistry of fluorescent molecules used in the reaction.

During the last decade, different fluorescent molecules were used to detect and quantify DNA amplification in 'real-time'. Among these, SYBR Green[3] provided the simplest method for detecting and quantitating PCR products in real-time reactions with high sensitivity. SYBR Green binds to double-stranded DNA and emits light upon excitation. As the reaction proceeds and the PCR products accumulate, the fluorescence increases proportional to the amount of specific DNA present in the original sample. As the amplification starts, more SYBR Green molecules are associated with the newly synthesized double-stranded DNA and the fluorescenece steadily increases. This enables the detection of template DNA present in femtogram levels! The problem of sensitivity was finally solved.

[3]SYBR Green has affinity for only double-stranded DNA and emits 1000-fold greater florescence when it is associated with the minor groove of double-stranded DNA than when it is free in solution [3]. SYBR Green is added in the PCR reaction along with other reactants and a laser detector is used to detect the level of fluorescence.

Analysis of Real-Time PCR data

As shown in *Figure* 2, in a typical Real-time PCR reaction, the reaction mixture containing template, polymerase, primers, *etc*., is mixed with SYBR Green. As the number of cycle progresses, the amount of double-stranded DNA increases and so does the fluorescence. To quantitate the amount of DNA template, the software of the machine plots the Number of Cycles versus Laser reading of Fluorescence Intensity. This graph is known as 'Amplification Curve' (*Figure* 4). A threshold is decided depending upon the range of detection of amplification. As soon as the fluorescence crosses that threshold, the software is confident that amplification has occurred. The cycle at which fluorescence crosses threshold value is called 'Cycle threshold' or 'Ct'.

Analysis of Ct value can allow important interpretations. For example, if initially the serum has 10 viral genomes, it will reach Ct, say in 30 cycles. But if there are 100 viral genomes, then it will reach Ct in a fewer number of cycles, say 10. Thus, the more the Ct value, the lower the viral genome in the serum sample.

This joy of solving the quantification problem was, however, short lived. Another problem cropped up. The problem of non-specific products (generated as a result of the primers binding

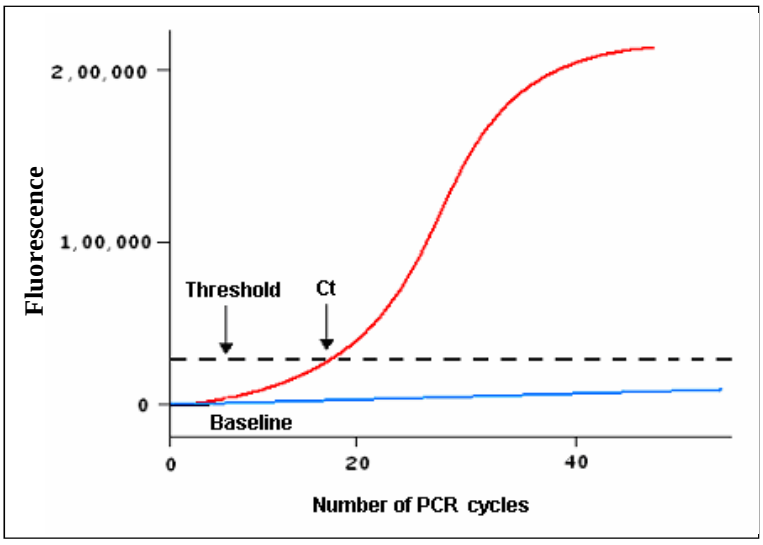

Figure 4. Representative graph of Real-Time PCR data. Baseline (Blue): No amplification curve. (Red): Amplification curve.

non-specifically to the template DNA) has haunted PCR for a long time. The SYBR Green molecule can also bind to these non-specific double-stranded DNA and primer-dimer complexes. This came as a big blow to Real-time PCR methodology. But as it has been rightly said, *there is always a way out*. This problem was countered by the introduction of 'dissociation-curve analysis'. Again, chemistry of DNA came to the rescue. The melting temperature of DNA depends upon the length of the strand and its GC[4] content. These properties can be exploited to distinguish between the specific and non-specific products. Thus, an extra step was added to the PCR reaction. After the reaction is complete, the amplification products are cooled to 25 °C and then the temperature of the solution is slowly but progressively increased again. The laser now detects the loss of fluorescence as more of SYBR Green is released when DNA melts at high temperature. As non-specific products are likely to have different lengths and GC content compared to the specific product, they usually melt at different temperatures compared to the specific amplified product. A 'Dissociation curve' (*Figure* 5) is a graph of 'Temperature vs Fluorescence'. To ascertain whether the peak obtained is of desired product, the value can be compared with standards.

[4]DNA double strands with higher GC content melt at higher temperature than DNA having a higher AT content.

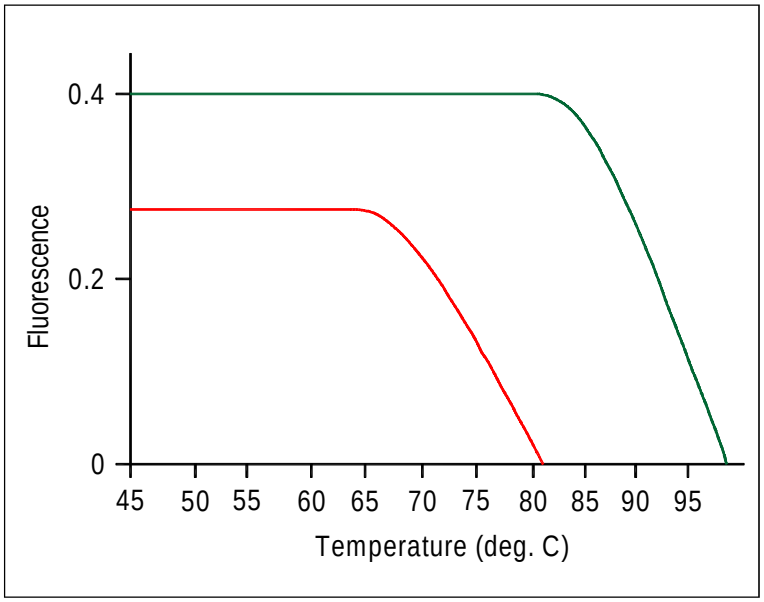

Figure 5. Dissociation curve. DNA sample A (red) and sample B (green) give different peaks due to difference in the GC content as well as length. Sample A dissociates much before sample B, suggesting that sample A has low GC content and lesser length than sample B.

Traditional PCR	Real-Time PCR
Post-PCR processing is needed	DNA is quantified during the reaction
Low Sensitivity	High Sensitivity
The range of detection is very low (about 2 log)	The range of detection is high (multiple log)
Semi-quantitative at best	**Can be used for quantification**
Size of amplified DNA ranges from about 100 base pairs to 3 kilo base pairs	Amplicon size is generally 150-300 base pairs. Small size is best for normal assay conditions. Reaction conditions need to be changed to obtain amplicons with large sizes.

Table 1. Differences Between Traditional and Real-time PCR.

This method altogether reduced the need for electrophoresis to check the amplified product. The dissociation curve analyses can differentiate between two different peaks for two different DNA molecules of same size, which would otherwise give a band at the same position in gel electrophoresis. Differences between traditional and Real-time PCR are summarized in *Table* 1.

Applications of Real-Time PCR

The sensitivity and specificity of Real-time PCR has made it a powerful tool both for basic research and for medical diagnostics. In biological research, the most important use of Real-Time PCR is the relative and absolute quantitation of gene expression under various conditions. For example, if a gene is expressed in higher amount in cancer tissue, Real-time PCR can detect this differential expression. Early detection of such expression profile can determine the predisposition of certain individual to different diseases like diabetes.

Since the emergence of this promising technology, all molecular diagnostic approaches have converged on the Real-time PCR

system (4). It has drastically decreased the time frame of clinical trials pertaining to drug resistance as well as testing the effectiveness of drugs against elimination of pathogen and has been effectively used to check drug resistance in HIV, which has resulted in faster screening of alternative drugs. It is extremely useful in detection and identification of bacterial strains. Immediate and specific detection offers the advantage of prescribing highly specific antibiotics rather than a broad mixture of antibiotics, which may result in development of antibiotic resistant strains, and has also been very effective in identification of specific DNA sequences in clinical oncology.

Those days are history when symptoms were used to diagnose the onset of a disease. Today we can screen samples before the symptoms show up in susceptible individuals and can improve their quality of life due to early detection.

Suggested Reading

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