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Real Time And Traditional Polymerase Chain Reaction: A Comparative Study

Simran Singh, Saurabh Rawat, Okesh Chabbra

Abstract: Real-Time PCR—also called quantitative polymerase chain reaction (qPCR)—is one of the most powerful and sensitive gene analysis techniques available and is used for a broad range of applications including quantitative gene expression analysis, genotyping, SNP analysis, pathogen detection, drug target validation and for measuring RNA interference. Frequently, real-time polymerase chain reaction is combined with reverse transcription to quantify messenger RNA (mRNA) and MicroRNA (miRNA) in cells or tissues. Traditional methods use Agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction. To understand why traditional PCR is limiting, it is important to understand what happens during a PCR reaction.

Index Terms: Formulation, Zone of Inhibition, Antibiotic Sensitivity Test.

1 INTRODUCTION

1.1 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) has been invented in 1983 by Kary Mullis (Nobel Price in 1993), (Mullis K. et Fobona F., 1987). Three years after its invention, there was an incredible expansion of its use thanks to the commercialization of the Taq polymerase, a polymerase that resists high temperatures. The principle, and aim, of the PCR technology is to specifically increase a target from an undetectable amount of starting material. In classical PCR, at the end of the amplification, the product can be run on a gel for detection of this specific product. In Real-Time PCR, this step can be avoided since the technology combines the DNA amplification with the immediate detection of the products in a single tube. The homogeneous format is highly beneficial as it removes the significant contamination risk caused by opening tubes for post-PCR manipulation. It is also less time consuming than gel based analysis and can give a quantitative result.

1.1.1 A basic PCR set up requires several components and reagents. These components include:

- DNA template that contains the DNA region (target) to be amplified.
- Two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target.
- Taq polymerase or another DNA polymerase with a temperature optimum at around 70 °C.
- Deoxynucleoside triphosphates (dNTPs, sometimes called "deoxynucleotide triphosphates"; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand.
- Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- Divalent cations, magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis[9]
- Monovalent cation potassium ions.

1.2 Real time PCR

As the name suggests, real-time PCR measures PCR amplification as it occurs. This completely revolutionizes the way one approaches PCR-based quantitation of DNA and RNA. Real-time Polymerase Chain Reaction (PCR) is the ability to monitor the progress of the PCR as it occurs (i.e., in real time). Data is therefore collected throughout the PCR process, rather than at the end of the PCR. This completely revolutionizes the way one approaches PCR-based quantitation of DNA and RNA. In real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. In contrast, an endpoint assay (also called a “plate read assay”) measures the amount of accumulated PCR product at the end of the PCR cycle. Real-Time PCR focuses on the exponential phase because it provides the most precise and accurate data for quantitation. Within the exponential phase, the real-time PCR instrument calculates two values. The Threshold line is the level of detection at which a reaction reaches a fluorescent intensity above background. The PCR cycle at which the sample reaches this level is called the Cycle Threshold, Ct. The Ct value is used in downstream quantitation or presence/absence detection. By comparing the Ct values of samples of unknown concentration with a series of standards, the amount of template DNA in an unknown reaction can be accurately determined.

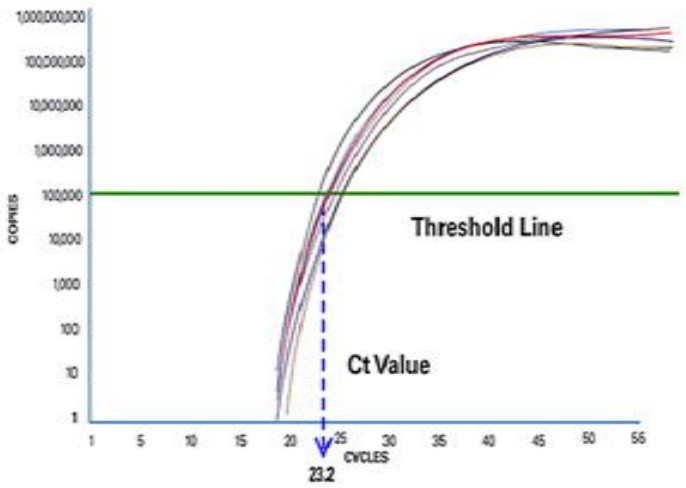

Fig. 1: The PCR cycle at which the sample reaches a fluorescent intensity above background is the Cycle Threshold or Ct.

1.3 Traditional PCR

In traditional PCR, results are collected after the reaction is complete, making it impossible to determine the starting concentration of nucleic acid. Traditional PCR Measures at the Plateau, Giving You Variable Results. In Figure 2, three replicate samples, which had same amount of DNA in the beginning of the reaction, have different quantities of PCR product by the plateau phase of the reaction (due to variations in reaction kinetics). Therefore, it will be more precise to take measurements during the exponential phase, where the replicate samples are amplifying exponentially.

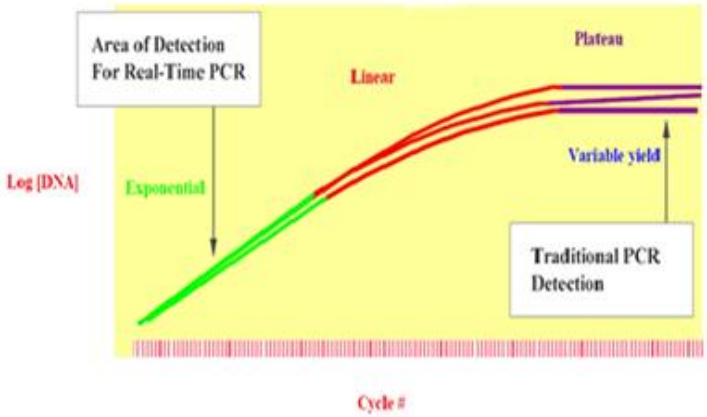

Fig. 2: Identical Samples Produce Different Quantities of Reaction Product by the Plateau Phase of PCR

2 METHODOLOGY

2.1 Formulation of Herbal Sanitizer

By The PCR reaction follows the three main steps:

- **Denaturation** - This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.
- **Annealing** - The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3–5 °C below the Tm of the primers used. Stable DNA–DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.
- **Extension** - The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C,[12][13] and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified.

1.1.3 A basic PCR run can be broken up into three phases:

Exponential

Exact doubling of product is accumulating at every cycle (assuming 100% reaction efficiency). The reaction is very specific and precise. Exponential amplification occurs because all of the reagents are fresh and available, the kinetics of the reaction push the reaction to favor doubling of amplicon.

Linear (High Variability)

As the reaction progresses, some of the reagents are being consumed as a result of amplification. The reactions start to slow down and the PCR product is no longer being doubled at each cycle.

Plateau (End-Point: Gel detection for traditional methods) The reaction has stopped, no more products are being made and if left long enough, the PCR products will begin to degrade. Each tube or reaction will plateau at a different point, due to the different reaction kinetics for each sample. These differences can be seen in the plateau phase. The plateau phase is where traditional PCR takes its measurement, also known as end-point detection.

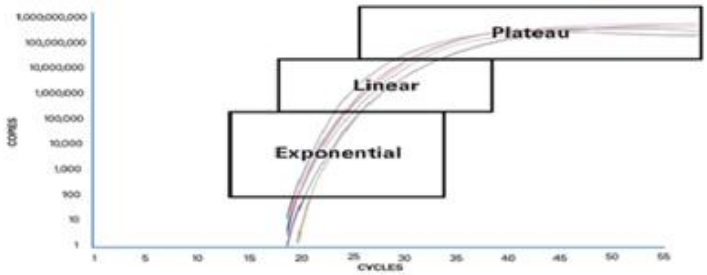

Fig. 3: PCR Phases

3 RESULTS

TABLE 1
COMPARISON BETWEEN DIFFERENT PCR

	Digital PCR	Real Time PCR	Traditional PCR
Overview	Measures the fraction of negative replicates to determine absolute copies.	Measures PCR amplification as it occurs.	Measures the amount of accumulated PCR product at the end of the PCR cycles.
Quantitative?	Yes, the fraction of negative PCR reactions is fit to a Poisson statistical algorithm.	Yes, because data is collected during the exponential growth (log) phase of PCR when the quantity of the PCR product is directly proportional to the amount of template nucleic acid.	No, though comparing the intensity of the amplified band on a gel to standards of a known concentration can give you 'semi-quantitative' results.
Applications	• Absolute Quantification of Viral Load • Absolute Quantification of Nucleic Acid Standards • Absolute Quantification of Next-Gen Sequencing Libraries • Rare Allele Detection • Absolute quantification of gene expression • Enrichment and Separation of Mixtures	• Quantitation of Gene Expression • Microarray Verification • Quality Control and Assay Validation • Pathogen detection • SNP Genotyping • Copy Number Variation • MicroRNA Analysis • Viral Quantitation • siRNA/RNAi experiments	Amplification of DNA for: • Sequencing • Genotyping • Cloning

	Advantages of digital PCR	Advantages of Real Time PCR	Disadvantages of Traditional PCR
Summary	• No need to rely on references or standards Desired precision can be achieved by increasing total number of PCR replicates • Highly tolerant to inhibitors • Capable of analyzing complex mixtures. • Unlike traditional qPCR, digital PCR provides a linear response to the number of copies present to allow for small	• Increased dynamic range of detection • No post-PCR processing • Detection is capable down to a 2-fold change • Collects data in the exponential growth phase of PCR • An increase in reporter fluorescent signal is directly proportional to the number of amplicons generated. • The cleaved probe provides a permanent record amplification of an amplicon	• Poor Precision • Low sensitivity • Short dynamic range < 2 logs • Low resolution • Non-Automated • Size-based discrimination only • Results are not expressed as numbers • Ethidium bromide for staining is not very quantitative • Post-PCR processing

4 CONCLUSION

Real-Time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection. Traditional methods use Agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction. Agarose gel results are obtained from the end point of the reaction. Endpoint detection is very time consuming. Results may not be obtained for days. Results are based on size discrimination, which may not be very precise. The end point is variable from sample to sample. While gels may not be able to resolve these variabilities in yield, real-time PCR is sensitive enough to detect thes changes. Agarose Gel resolution is very poor, about 10 fold. Real-Time PCR can detect as little as a two-fold change!

References

[1] http://www6.appliedbiosystems.com/support/tutorials/pdf/rtpcr_vs_tradpcr.pdf

[2] http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_042485.pdf

[3] http://www.eurogentec.com/uploads/qPCR-guide.pdf

[4] http://www.viracoribt.com/Learning-Lab/PCR

[5] pathmicro.med.sc.edu/pcr/realtime-home.htm

[6] www.lifetechnologies.com/in/en/home/life.../pcr/real-time-pcr.html

[7] www.bio.davidson.edu/courses/molbio/molstudents/.../realtimepcr.htm

[8] qpcr.gene-quantification.info/