The Polymerase Chain Reaction and real-time quantitative RT-PCR

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The Polymerase Chain Reaction

The following information is sourced from http://www.ncbi.nlm.nih.gov/probe/docs/techpcr/, under the copyright guidelines reproduced at the bottom, and supplemented with information from Wikipedia.

Introduction

The PCR (Polymerase Chain Reaction) is a technology in molecular biology used to massively amplify a single copy or a few copies of a piece of DNA to generate thousands to millions of copies of a particular DNA sequence.

It is now a common and often indispensable technique used in medical and biological research labs for a variety of applications that include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity

testing); and the detection and diagnosis of infectious diseases.

It was developed by Kary Mullis in the 1980s and in 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR.

PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand.

- Because DNA polymerase can add a nucleotide only onto a pre-existing 3′-OH group, it needs a primer to which it can add the first nucleotide.
- This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify.
- At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons).

The set of slides below explains this technique very well. It is sourced from the Biochemical Methods Lab Manual of the College of Chemistry and Biochemistry, The University of Oklahoma

(http://www.ou.edu/OpenEducation/ou-resources/biochemical-methods/) under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 license.

Polymerase Chain Reaction (PCR) Lecture from **ouopened**

Components of PCR

DNA template – the sample DNA that contains the target sequence. At the beginning of the reaction, high temperature is applied to the original double-stranded DNA molecule to separate the strands from each other.

DNA polymerase – a type of enzyme that synthesizes new strands of DNA complementary to the target sequence.

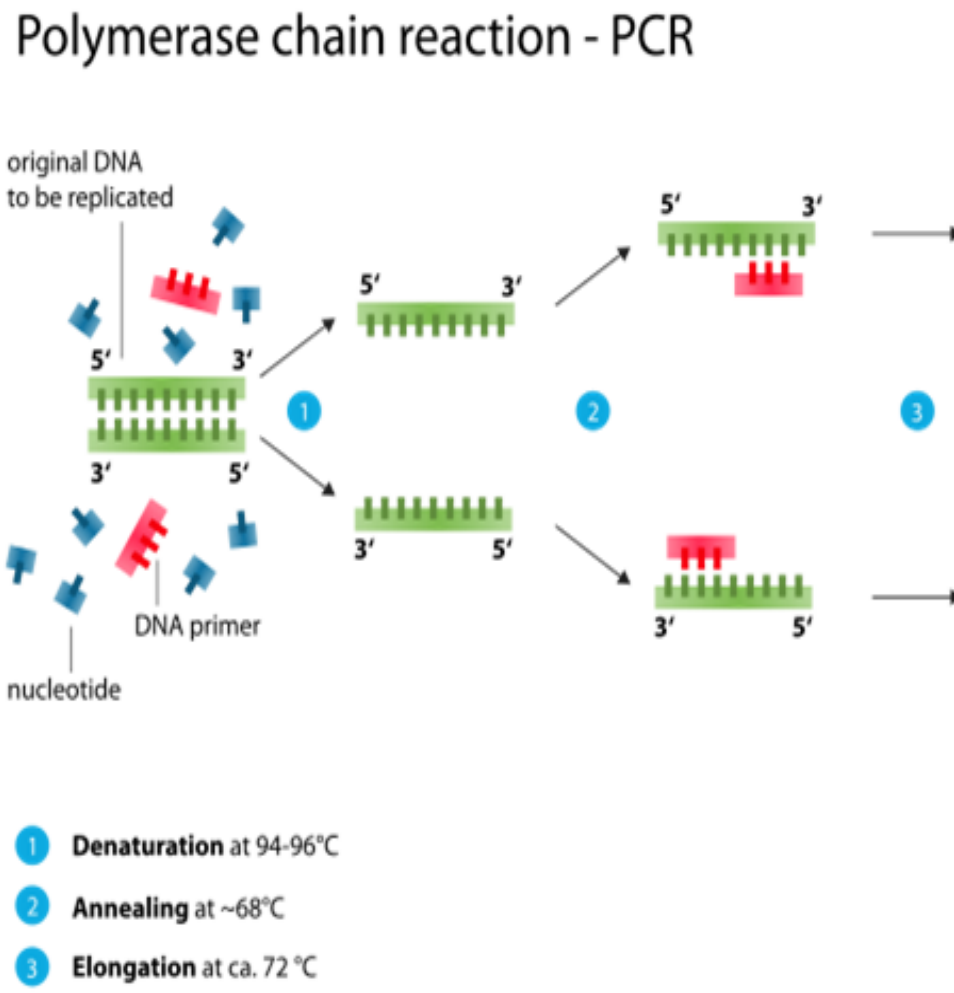
- The first and most commonly used of these enzymes is *Taq* DNA polymerase (from *Thermis aquaticus*), whereas *Pfu* DNA polymerase (from *Pyrococcus furiosus*) is used widely because of its higher fidelity when copying DNA.
- Although these enzymes are subtly different, they both have two capabilities that make them suitable for PCR: 1) they can generate

new strands of DNA using a DNA template and primers, and 2) they are heat resistant.

Primers – short pieces of single-stranded DNA that are complementary to the target sequence. The polymerase begins synthesizing new DNA from the end of the primer.

Nucleotides (dNTPs or deoxynucleotide triphosphates) – single units of the bases A, T, G, and C, which are essentially "building blocks" for new DNA strands.

RT-PCR (Reverse Transcription PCR) – PCR preceded with conversion of sample RNA into cDNA with enzyme **reverse transcriptase.**



https://www.youtube.com/watch?v=c-f1H07D_70

Limitations of PCR and RT-PCR

The PCR reaction starts to generate copies of the target sequence exponentially. Only during the exponential phase of the PCR reaction is it possible to extrapolate back to determine the starting quantity of the target sequence contained in the sample. Because of inhibitors of the polymerase reaction found in the sample, reagent limitation,

accumulation of pyrophosphate molecules, and self-annealing of the accumulating product, the PCR reaction eventually ceases to amplify target sequence at an exponential rate and a "plateau effect" occurs, making the end point quantification of PCR products unreliable. This is the attribute of PCR that makes Real-Time Quantitative RT-PCR so necessary

Demonstration of the steps of the PCR

Here is a very nice video taking you through the steps of the PCR.

https://drive.google.com/file/d/1BbjUSybq6KX1Q-WuRKw3-ZdHaY0bFogD/view?usp=sharing

Real-Time qRT-PCR

Introduction

Real-Time Quantitative Reverse Transcription PCR is a major development of PCR technology that enables reliable detection and measurement of products generated during each cycle of PCR process.

- This technique became possible after introduction of an oligonucleotide probe which was designed to hybridize within the target sequence.

- Cleavage of the probe during PCR because of the 5′ nuclease activity of Taq polymerase can be used to detect amplification of the target-specific product.

Techniques to monitor degradation of the probe

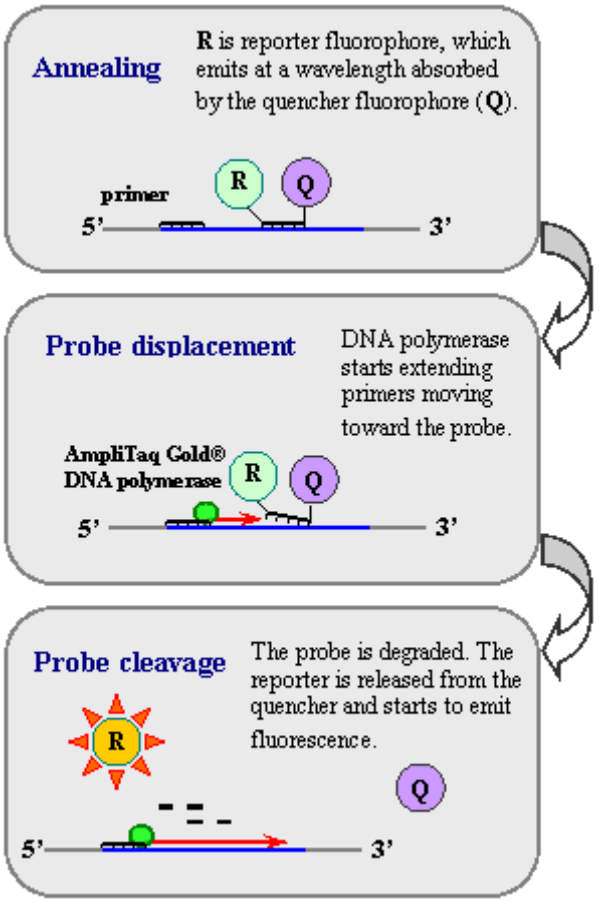
- Intercalation of double-stranded DNA-binding dyes
- $_{32}P$ probe labeling
- Labeling of the probe with fluorescent dyes

TaqMan

This assay (named after *Taq* DNA polymerase) was one of the earliest methods introduced for real time PCR reaction monitoring and has been widely adopted for both the quantification of mRNAs and for detecting variation.

- The method exploits the 5′ endonuclease activity of *Taq* DNA polymerase to cleave an oligonucleotide probe during PCR, thereby generating a detectable signal.
- The probes are fluorescently labeled at their 5′ end and are non-extendable at their 3′ end by chemical modification.
- Specificity is conferred at three levels: via two PCR primers and the probe.

- Applied Biosystems probes also include a minor groove binder for added specificity.



Nomenclature used in RT-qRT-PCR

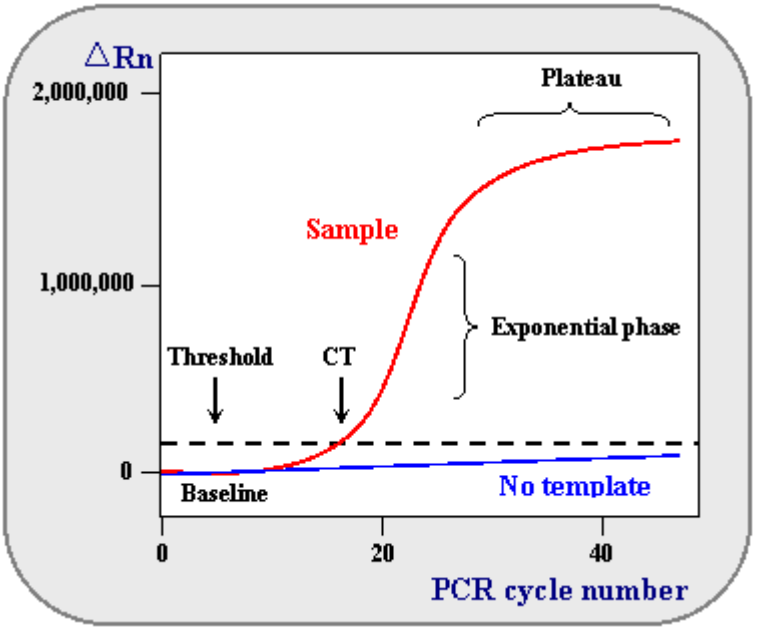
Baseline is defined as PCR cycles in which a reporter fluorescent signal is accumulating but is beneath the limits of detection of the instrument.

ΔRn is an increment of fluorescent signal at each time point. The ΔRn values are plotted versus the cycle number.

Threshold is an arbitrary level of fluorescence chosen on the basis of the baseline variability. A signal that is detected above the threshold is

considered a real signal that can be used to define the threshold cycle (Ct) for a sample. Threshold can be adjusted for each experiment so that it is in the region of exponential amplification across all plots.

Ct is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold. The Ct is a basic principle of real time PCR and is an essential component in producing accurate and reproducible data.



Applications of Real Time Quantitative RT-PCR

- Relative and absolute quantification of gene expression.
- Validation of DNA microarray results.
- Variation analysis including SNP discovery and validation.
- Counting bacterial, viral, or fungal loads, etc.

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https://www.youtube.com/watch?v=aUBJtHwHASA