

Beginner's guide to Real-time PCR



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PCR or the Polymerase Chain Reaction has become the cornerstone of modern molecular biology the world over. Real-time PCR is a bespoke form of the Polymerase Chain Reaction that maximizes the potential of the technique.

To understand Real Time PCR it's easier to begin with the principles of a basic PCR

Principles of PCR

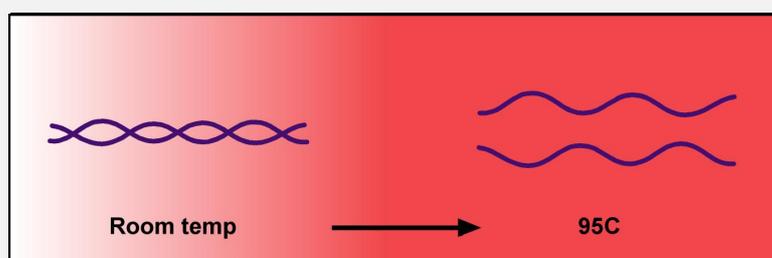
PCR is a technique for amplifying DNA. There are 2 reasons why you may want to amplify DNA. Firstly you may want to simply create multiple copies of a rare piece of DNA. For example a forensic scientist may want to amplify a tiny piece of DNA from a crime scene. More commonly however you may wish to compare 2 different samples of DNA to see which is the more abundant. Because DNA is microscopic you cannot see which sample contains the most DNA. However, if you amplify both samples at the same rate, you can calculate which sample was the biggest to begin with by establishing which is the biggest after amplification.

It is a Polymerase enzyme that drives a PCR. A polymerase will synthesize a complementary sequence of bases to any single strand of DNA providing it has a double stranded starting point.

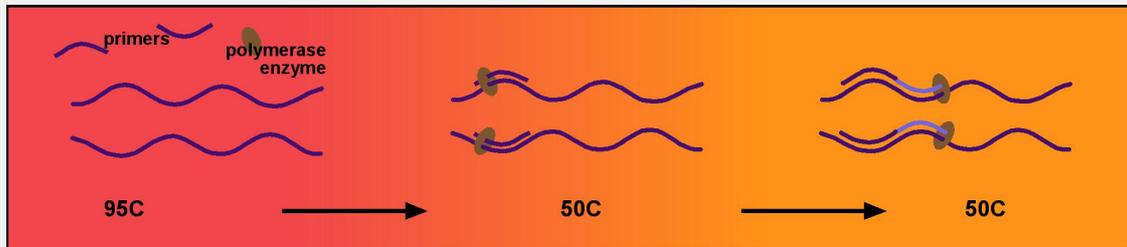
This is very useful because you can choose which gene you wish the polymerase to amplify in a mixed DNA sample by adding small pieces of DNA complimentary to your gene of interest. These small pieces of DNA are known as primers because they prime the DNA sample ready for the polymerase to bind and begin copying the gene of interest.

During a PCR, changes in temperature are used to control the activity of the polymerase and the binding of primers.

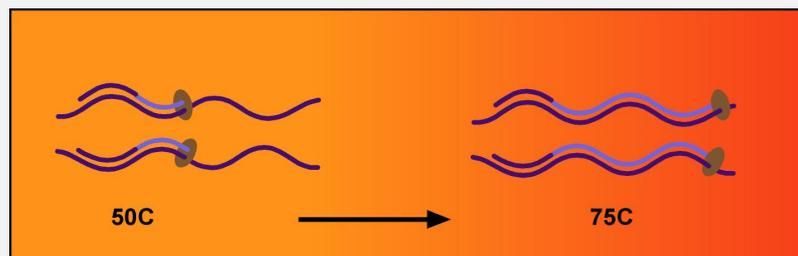
To begin the reaction the temperature is raised to 95°C. At this temperature all double stranded DNA is "melted" in to single strands:



The temperature is then lowered to $\sim 50^{\circ}\text{C}$. This allows the primers to bind to your gene of interest. Thus the polymerase has somewhere to bind and can begin copying the DNA strand:

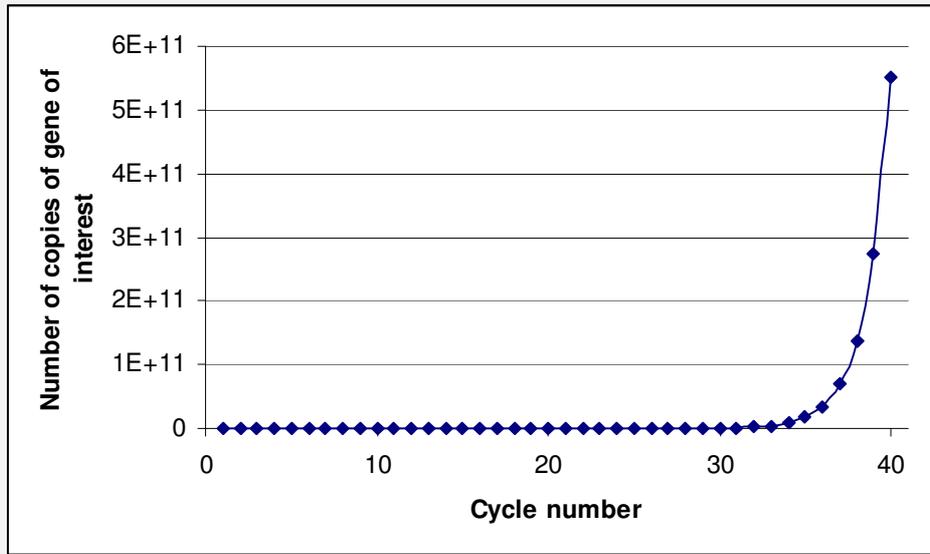


The optimal temperature for the polymerase to operate is 72°C so at this point the temperature is sometimes raised to 72°C to allow the enzyme to work faster. There are now twice as many copies of your gene of interest as when you started:



The cycle of changing temperatures (95°C , 50°C and 72°C) is then repeated and two copies become four. Another cycle and four become eight, and so on... for 40-50 cycles.





After amplifying your gene in to many millions of copies it is possible to run the amplified DNA out on a polyacrilamide gel and stain it with a dye which makes is visible. The bigger the visible band, the more copies of your gene you have created.

Hence if you are comparing 2 samples e.g. one from a healthy patient and one from a cancer patient, you can see in which sample your gene of interest was expressed most highly.

Real-Time PCR

Real-Time PCR is identical to a simple PCR except that the progress of the reaction is monitored by a camera or detector in "real-time".

There are a number of techniques that are used to allow the progress of a PCR to be monitored. Each technique uses some kind of fluorescent marker which binds to the DNA. Hence as the number of gene copies increases during the reaction so the fluorescence increases. This is advantageous because the efficiency and rate of the reaction can be seen. There is also no need to run the PCR product out on a gel after the reaction.

Methods of monitoring DNA amplification in "real-time":

Fluorescent dyes

Intercalating fluorescent dyes (e.g. SYBR green) are the simplest and cheapest way to monitor a PCR in real-time. These dyes fluoresce only when bound to double-stranded DNA. So as the number of copies of DNA increases during the reaction so the fluorescence increases. The major disadvantage of using a dye such as this is the lack

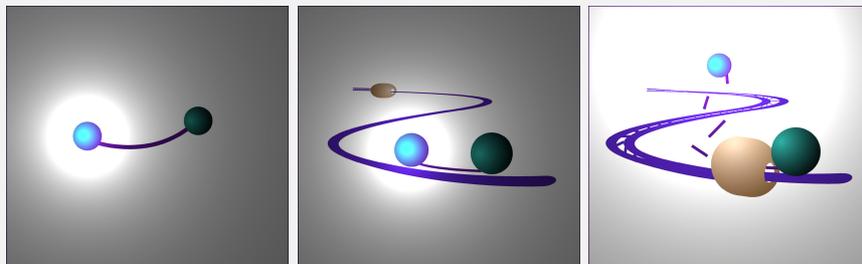


of specificity. This dye will report the amplification of any DNA not just your gene of interest.

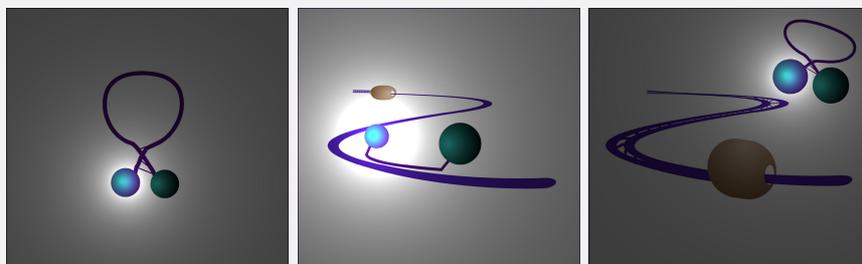
Fluorescent probes

Fluorescent probes are pieces of DNA complimentary to your gene of interest that are labeled with a fluorescent dye.

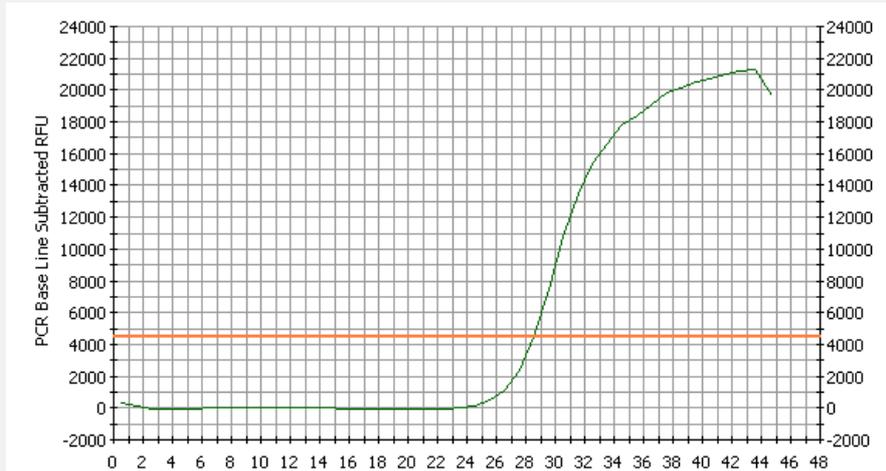
The simplest and most commonly used type of probe is the Taqman-type probe. These probes are labeled with a fluorescent reporter molecule at one end and a quencher molecule (capable of quenching the fluorescence of the reporter) at the other. Hence under normal circumstances the fluorescent emission from the probe is low. However during the PCR the probe binds to the gene of interest and becomes cleaved by the polymerase. Hence the reporter and quencher are physically separated and the fluorescence increases.



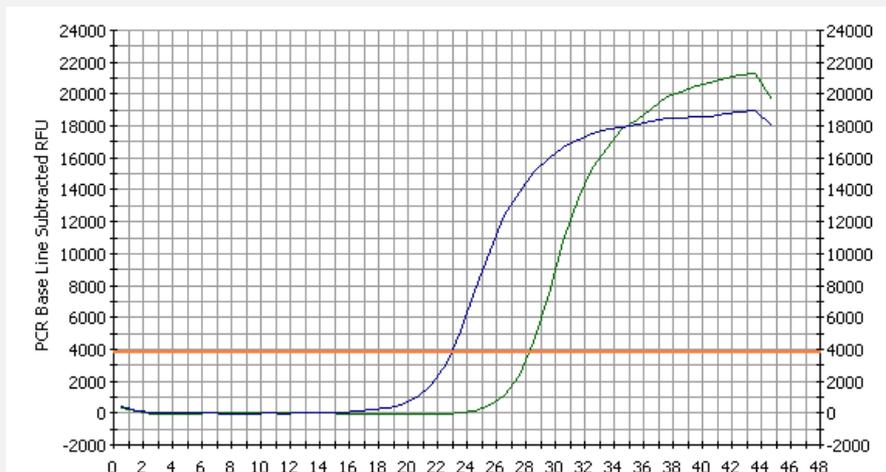
Another commonly used type of probe is the “molecular beacon”. Again these are small pieces of DNA complimentary to your gene of interest labeled with a fluorescent reporter and a quencher molecule on opposite ends. These probes are designed to fold on to themselves to bring the reporter and quencher in to closer proximity and minimize fluorescent emission. However, when the probe binds to the gene of interest the probe takes up a linear conformation and the reporter and quencher are separated. This results in the desired increase in fluorescence. Molecular beacon probes are not cleaved by the polymerase but are simply “knocked off” again.



The output from a real-time PCR reaction is in the form of a graph showing the number of PCR cycles (1 cycle = 90°C, 50°C, 72°C) against the increasing fluorescence. This is known as an amplification plot:



The horizontal line on the graph represents a “threshold” set by the user. The point at which the amplification plot crosses this threshold is known as the Ct (cross threshold) value. Logic dictates that the lower the Ct value for a sample the greater the starting amount of DNA in the sample. Thus if two amplification plots are compared it is simple to deduce which sample contained the greatest amount of the DNA of interest by the Ct value:



Blue sample Ct value=23. Green sample Ct value=28. Therefore the blue sample contained 32 (2^5) times more of the gene of interest than the green sample.



Kits, reagents and expertise

Real-Time PCR is an exceptionally powerful research tool. With the correct kits, reagents and experimental design it is quick and easy to generate high quality meaningful data in no time with real-time PCR.

Whether you are an expert or a complete beginner PrimerDesign Ltd can make your life very easy. We are experts and can help you by providing everything you need to generate the very best possible real-time PCR data.

What will you need?

Precision Reverse Transcription kit



The PrimerDesign Precision Reverse Transcription kit is used to reverse transcribe RNA from your biological samples in to cDNA ready for real-time PCR.

Real-time PCR primers (and probe)



PrimerDesign can design, synthesise, validate and optimise real-time PCR primers for you for any gene in any species. They will be shipped to you guaranteed to give your great data on the day they arrive

Housekeeping primers



Measuring a "housekeeping gene" or "endogenous control" gene is the commonest strategy for normalising real-time PCR data. PrimerDesign can supply high quality housekeeping gene assays for any species.

geNorm kit



You might like to rationally select which housekeeping genes to use in your experiment with a geNorm housekeeping gene selection kit.

Precision MasterMix



A high quality, cost saving mix containing all of the components needed to run the perfect real-time PCR reaction.



Here to help

Please don't hesitate to contact us if you are getting started in real-time PCR and would like some help. We have an expert friendly team who take a real pleasure in helping out our customers. There will be no waiting in queues. You'll get straight through to one of our expert team who will be delighted to chat about your work and point you in the right direction.

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