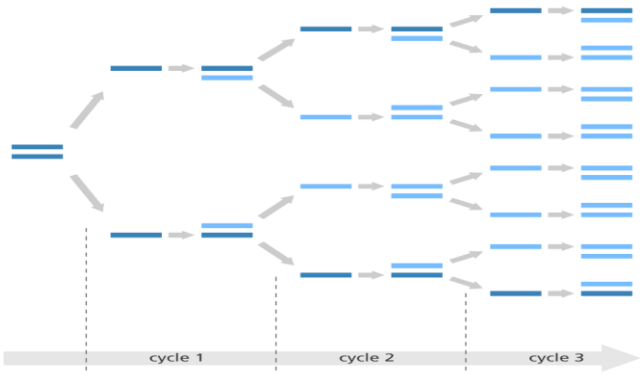
Polymerase Chain Reaction-361 BCH



1-Polymerase Chain Reaction

Nucleic acid amplification is an important process in biotechnology and molecular biology and has been widely used in research, medicine, agriculture and forensics. In order to study individual genes or specific DNA regions of interest, it is often necessary to obtain a large quantity of nucleic acid for study, rather than isolate a single copy of the target DNA from a large number of cells, it is often more useful to generate multiple copies of a target from a single molecule of DNA or mRNA, via an in vitro amplification method.

There are different methods developed for nucleic acid amplification, Polymerase chain reaction (PCR) was a method which was developed and until now has been the method of choice since its invention because of its simplicity, easier methodology, extensively validated standard operating procedure and availability of reagents and equipments.

The PCR is used to amplify a precise fragment of DNA from a complex mixture of starting material termed the template DNA which controlled by heating and cooling. It does require the knowledge of some DNA sequence information which flanks the fragment of DNA to be amplified (target DNA). From this information two oligonucleotide primers may be chemically synthesised each complementary to a stretch of DNA to the 30 side of the target DNA, one oligonucleotide for each of the two DNA strands (DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group). It may be thought of as a technique analogous to the DNA replication process that takes place in cells since the outcome is the same: the generation of new complementary DNA stretches based upon the existing ones. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons). In only 20 cycles, PCR can product about a million (2^{20}) copies of the target.

The polymerase chain reaction has been elaborated in many ways since its introduction and is now commonly used for a wide variety of applications including genotyping, cloning, mutation detection, sequencing, microarrays, forensics, and paternity testing.

2-Components of PCR

Table 4-1 shows the essential component of any PCR reaction, and their important

Components	Function
Template DNA	The template carries the DNA segment, or target, you wish to amplify.
forward and reverse primers,	A primer is a short, single-stranded piece of DNA that anneals (attaches) to its complementary sequence on the template. A pair of primers will bind to either side of the target DNA segment providing initiation sites for DNA synthesis.

Taq polymerase	This is the enzyme used to synthesize new strands of DNA. DNA polymerase adds nucleotides onto the end of an annealed primer.
deoxynucleotide triphosphates (dNTPs)	These are the four nucleotides used by DNA polymerase to extend an annealed primer
$MgCl_2$	DNA polymerase requires magnesium for activity. Magnesium is usually supplied to a PCR amplification in the form of magnesium chloride.

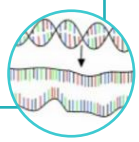
- **Additional reagents may include, Potassium salt K+, dimethylsulfoxide (DMSO) , formamide, bovine serum albumin or Betaine.**

3-PCR Steps

PCR proceeds in three distinct steps <u>Governed by Temperature</u>:

- the double-stranded template DNA is denatured by heating, typically to 95°C, to separate the double stranded DNA

Denaturation: (95°C)

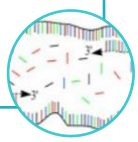


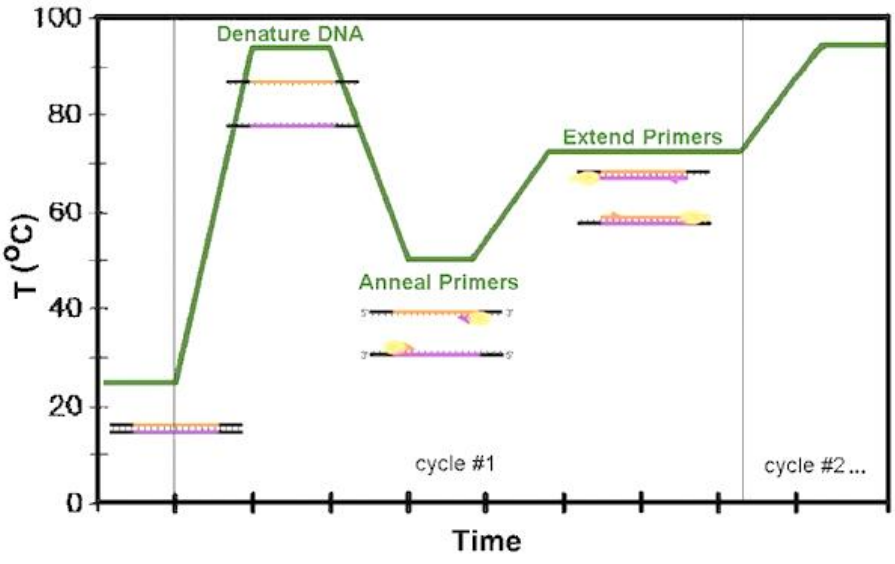
- the reaction is rapidly cooled to an annealing temperature to allow the oligonucleotide primers to hybridize to the template.

****Annealing:(50-65°C)**

- the reaction is heated to a temperature, typically 72°C for efficient DNA synthesis by the thermostable DNA polymerase.

Exention:(72°C)





*** *Note on Annealing Temperature:**

The single strands of the template are too long and complex to be able to reanneal during this rapid cooling phase. During this annealing step the thermostable DNA polymerase will be active to some extent and will begin to extend the primers as soon as they anneal to the template. The annealing temperature chosen for a PCR depends directly on length and GC composition of the primers.

Performing PCR:

Primer Design

To design a primer, many parameters should be considered:

- Primer length should be 15-30 nucleotide residues (bases).
- Optimal G-C content should range between 40-60%.
- The 3' end of primers should contain a G or C
- The 3' ends of a primer set, should not be complementary to each other, nor can the 3' end of a single primer be complementary to other sequences in same the primer.
- Optimal melting temperatures (T_m) for primers range between 52-58 °C, although the range can be expanded to 45-65 °C. The final T_m for both primers should differ by no more than 5 °C.

- Di-nucleotide repeats (e.g., GCGCGCGCGC or ATATATATAT) or single base runs (e.g., AAAAA or CCCCC) should be avoided.

Important Links for primer design

NCBI Primer design tool http://www.ncbi.nlm.nih.gov/tools/primer-blast/
Primer3 http://frodo.wi.mit.edu/primer3/
In order to avoid amplification of related pseudogenes or homologs it could be useful to run a blast on NCBI to check for the target specificity of the primers. **http://blast.ncbi.nlm.nih.gov/Blast.cgi**

Performing and optimization of PCR

Generally, the concentration of PCR components should be as the following:

Table 4-2: concentrations of PCR components*

Component	Final concentration
Taq polymerase	0.5–2.0 units, ideally 1.25 units
Deoxynucleotides (dNTPs)	Typical concentration is 200 µM of each dNTP.
Magnesium Concentration	1.5-2.0 mM is optimal for Taq DNA Polymerase
Forward Primers	Typically 0.1-0.5 µM of each primer
Reverse Primer	Typically 0.1-0.5 µM of each primer
DNA Template	1ng–1µg of genomic templates

***(It depends on the target that will be amplified)**

Setting Up Thermal Cycling Conditions and optimizing the annealing temperature:

• Initial denaturation a typical reaction will start with a three minutes denaturation at 95 °C. (to denature the template).

• The next step is to set the thermal cycler to initiate a 25 to 35 cycles of a three-step temperature cycle (denaturation-annealing-extension).

• The final phase of thermal cycling incorporates an extended elongation period of 5 minutes or longer. This last step allows synthesis of many uncompleted amplicons to finish.

Table 4-3: PCR steps

Step	Temperature	Duration
Initial denaturation	95 °C	3 min
25-40 cycles of:		
Denaturation	95 °C	30 seconds
Annealing	50 °C to 60 °C	30 seconds
Extension	72 °C	1 min
Final Extension	72 °C	5 min

Optimizing annealing temperature

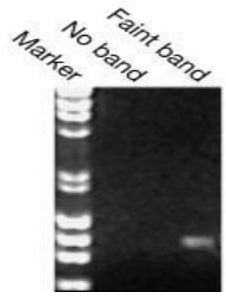
Optimize the annealing conditions by performing the reaction starting approximately 5°C below the calculated melting temperature of the primers and increasing the temperature for 8 degree Often fall in the range of 50-60°C.

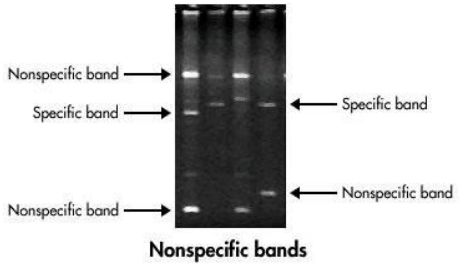
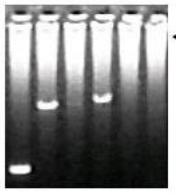
Post-PCR analysis

Once the PCR has finished, you need to analyze the products. The usual way of doing this is to size fractionate the DNA through an agarose gel. Examining the gel provides evidence for success or failure. The concentration of agarose depend on the product size.

PCR troubleshooting

If your PCR amplification somehow performs unexpectedly, it is usually caused by one of the listed possible error, depending on the error, try to change concentration, number of cycles….etc

Problem	Cause
No Band or Faint Band 	Too few cycles were used
	Incorrect annealing temperature
	Not enough template was in the reaction
	Primer concentration was too low
	Water was impure
Nonspecific Bands or Primer-Dimers	Too much primer was added
	Primers were designed or synthesized incorrectly by user or manufacturer
	Annealing temperature was too low
	Too many cycles were used

 Nonspecific bands	
Smeared Bands 	Too much template was added Primers contained impurities Template contained an exonuclease or was degraded

Important Links

Topic	link
Polymerase Chain Reaction	http://www.jove.com/video/3998/polymerase-chain-reaction-basic-protocol-plus-troubleshooting
PCR Animation	http://highered.mheducation.com/sites/0072556781/student_view0/chapter14/animation_quiz_6.html
PCR Troubleshooting	http://www.bio-rad.com/en-uk/applications-technologies/pcr-troubleshooting
History of PCR	http://siarchives.si.edu/research/videohistory_catalog9577.html

References:

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3831736/

http://oregonstate.edu/dept/biochem/hhmi/hhmiclasses/bb494/pcrohtwo.html

https://www.neb.com/tools-and-resources/usage-guidelines/guidelines-for-pcr-optimization-with-onetaq-and-onetaq-hot-start-dna-polymerases

http://www.bio-rad.com/en-uk/applications-technologies/pcr-troubleshooting