Polymerase Chain Reaction

The polymerase chain reaction (PCR) was developed by Kerry Mullis who won the Nobel Prize for this work. PCR can be used for gene cloning and manipulation, gene mutagenesis, DNA sequencing, forensic DNA typing and amplification of ancient DNA. Briefly, the principle is as follows. Short oligonucleotide primers are annealed to denatured DNA by using hybridization conditions ensuring that only primers with desired sequences will anneal. Two primers are complementary to the two 3' ends of DNA segment to be amplified, additionally; noncomplementary sequences at 5' ends such as a sequence to encode for restriction endonuclease cleavage can be added. Primers are extended using a DNA polymerase and the 4-deoxynucleotide triphosphates. A 3-step cycle is used that includes melting of DNA, annealing of primers and elongation of primers. This cycle is repeated more than 20 times to create a sufficient amount of the desired DNA.

Description of Components

- **PRIMERS**

  Typical primers are 18-28 nucleotides with 50-60% G+C content. Typical concentration in a PCR reaction is 0.1-0.5 mM; higher concentrations may give nonspecific products or primer dimers. Additionally, 3 or more C's or G's at 3' ends of primers may promote mispriming at G+C rich sequences. Furthermore, palindromic sequences within primers should be avoided; as should any internal inverted repeats that would cause primer to self anneal. Primers with a similar melting temperature that are completely complementary are recommended. The optimal melting temperature range for primers is 55^{o}C to 80^{o}C. An approximate melting value for your primer can be calculated before it is synthesized using the following equation: T_m (^{o}C) $\cong 2(N_A+N_T)+4(N_G+N_C)$.

- **DNA POLYMERASE**

  There are 2 common polymerases used for PCR, *Taq* and *Pfu* . The typical concentration is 2.5-5.0 units of

enzyme per 100 μL reaction for targets below 10 kb. Larger targets may require up to 10 units of enzyme per 100 μL of reaction volume. The most critical parameter affecting yield of PCR product is the extension time. *Taq* polymerase can amplify DNA faster than *Pfu* polymerase but it is not nearly as efficient. Normal extension time for *Pfu* polymerase is 2 min/kb of template whereas *Taq* polymerase can be as low as 0.5-1 min/kb. However, the mutated PCR product percentage per 1 kb fragment is 2.6% for *Pfu* polymerase and 16% for *Taq* polymerase. 72^{o}C is the optimal temperature for extension, as *Pfu* is most active and efficient under this condition.

- **REACTION BUFFER**

  Generally a 10-50 mM Tris/HCl buffer with a pH above 8.0 (typically 8.3-8.8). KCl can be added to facilitate primer annealing, but shouldn't be higher than 50 mM as this may inhibit polymerase. The deoxynucleotide triphosphates should have a total concentration of 0.4-1.0mM, meaning each dNTP is present in an equal amount (100-250 μM). Magnesium is also required and should have a higher concentration than the total dNTP concentration (0.5-2.5mM Mg^{2+}). Magnesium affects primer annealing and template denaturation, as well as enzyme activity and fidelity. An excess of magnesium gives nonspecific amplification products, while low magnesium yields lesser amount of desired product.

- **ADJUNCTS AND COSOLVENTS**

  Bovine serum albumin (BSA) can bind certain PCR inhibitors. The concentration range used can vary from 10-100μg/ml. Formamide (1.25-10%) facilitates primer-template annealing reactions and lowers the denaturing temperatures of melt resistant DNA. DMSO (1-10%) can improve denaturation of GC-rich DNA and help overcome difficulties of polymerase extension through secondary structure. Lastly, ammonium sulfate increases the ionic strength of reaction mixture, which alters denaturing and annealing temperatures of DNA and enzyme activity.

Reaction Conditions and Experimental Protocol

Temperature and length of time required for annealing depend upon base composition, length and concentration of amplification primers. Generally, the annealing temperature is 5^{o}C below the true melting temperature of the primers. Primers will anneal in a few seconds, for efficiency, higher

annealing temperatures can be used, which enhance the discrimination against incorrectly annealed primers. The annealing conditions need to be more stringent in the first 3 cycles to help increase specificity. If the temperature is lower than optimum additional DNA fragments are commonly observed. Denaturing conditions are best at 94-95^{o}C for 30-60 seconds. Lower temperatures may result in incomplete denaturation of target template and PCR products. Higher temperatures and a longer amount of time can lead to enzyme activity loss.

Diluting sample after first few rounds of PCR can be used to enhance PCR efficiency. This dilution may dilute potential inhibitors and the next round can use same primers or nested primers. In addition, the lowest number of cycles possible to achieve sufficient product should be used to assure a low number of errors. The order of addition of reaction mixture components is also of importance. *Pfu* polymerase has exonuclease activity and must be added last (i.e. after dNTP's), otherwise it may degrade primers. If primers and nucleotides are in the mixture at appropriate concentrations then primer degradation is minimal.

General PCR protocol

Prepare following mixture in appropriately sized eppendorf tube (0.5 mL or 0.2 mL):

81 μL of ddH_2O
10 μL of 10x polymerase buffer (for native of cloned *Pfu* polymerase)
2.5 μL of primer #1 (100 ng/μL)
2.5 μL of primer #2 (100 ng/μL)
1 μL of template DNA (<100 ng/μL)
1 μL of 10mM deoxynucleotide triphosphate mixture (2.5 mM each dNTP)
2 μL of DNA polymerase (native or cloned *Pfu* polymerase)

100 μL total reaction volume

Tap the side of the Eppendorf tube to mix the mixture properly, overlay it with drop of mineral oil and perform PCR reaction using following cycling parameters:

Segment	# of Cycles	Temperature	Duration
1	1	94-98^{o}C	45 sec
2	20-30	94-98^{o}C	45 sec
		Primer T_m-5^{o}C	45 sec
		72^{o}C	consider 2 min/kb of PCR target
3	1	72^{o}C	10 min

Troubleshooting

Observation	Possible cause	Solution
No product or low yield	Extension time too short	Recall 2 min/kb amplifying time of target
	Annealing temperature too high	Lowering annealing temperature in 5^{o}C increments
	Inappropriate buffer	Ensure appropriate buffer was used
	Order of addition of reaction components	Pfu DNA polymerase must be added last.
	GC content or secondary structure is too high	Use higher denaturation temperature or cosolvents such as DMSO or glycerol.
	Primer concentration too low	Check primer concentration
		Use high quality

	Non-priming or mis-priming	primers, check purity, melting, GC content and length
	Ionic strength of reaction mixture is too high	Remove extraneous salts
	Insufficient magnesium	Increase total magnesium to 2 mM
	Denaturation time too long	Reduce time or temperature of denaturation
	Poor template quality or template concentration too low	Use intact and highly purified templates at an adequate concentration.
Multiple Bands	Primer annealing too low	Increase the annealing temperature in $5^{o}C$ increments, use a hot start
	Non-specific primer to template annealing	Same as above
Artifactual smears on gel	Excessive amount of *Pfu* DNA polymerase	Decrease amount of DNA polymerase
	Extension time too long	Reduce extension time utilized

References

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