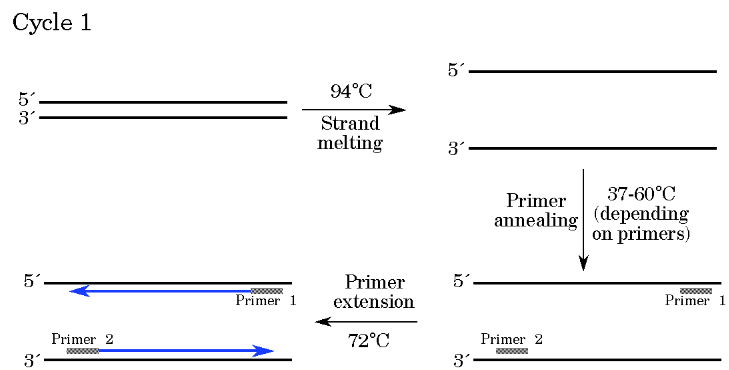
Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a technique that allows the generation of large amounts of a single DNA sequence from a mixture of sequences; the fragment generated can be designed to contain specific starting and ending positions based on the needs of the experiment.

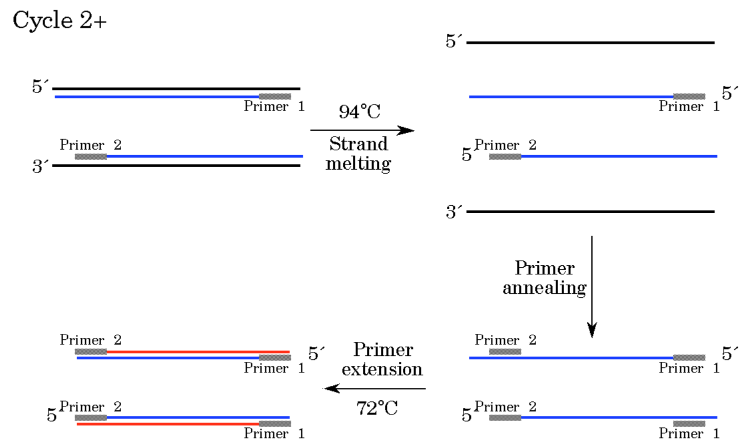
PCR uses a DNA polymerase to synthesize DNA. DNA polymerases require a "signal" to begin synthesis. The signal is a short fragment of DNA. PCR uses two synthetic oligonucleotides (known as primers) that correspond to (and thus base-pair to) the ends of the sequence of interest to act as synthesis initiation signals.

The two strands of the DNA template are separated by heating (usually to 94°C). The temperature is then decreased to allow the primers to bind to the template DNA. Once the primers have bound, the polymerase is allowed to synthesize new DNA strands (the polymerase most commonly used has a temperature optimum of 72°C.)

If the Cycle 1 reaction (the top of the figure at right) were the entire process, PCR would not be very useful. However, an examination of the figure reveals that, at the end of the first cycle, twice as much template DNA exists as was present at the beginning. Therefore, repeating the cycle allows the amount of product DNA to increase geometrically.



In theory, the amount of product will double each cycle. In practice, PCR is not quite that efficient, although it can produce tremendous quantities of DNA. It is literally possible to begin with a single molecule of DNA and generate enough DNA for any molecular biological technique. In addition, the DNA synthesized in the PCR reaction will have specific starting and ending points: the primer sequences define the end points of the fragments.



Good primers contain approximately 50% G+C and 50% A+T. This reduces problems in inducing template strand separation caused by the high affinity of G for C, and reduces non-specific priming common with high AT content.

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In addition, good primers have few regions of complementarity either internal to primer (*i.e.* secondary structure) or between two primers, especially at the 3´ end of the primers, and avoid repeated sequences (*e.g.*, AAAA or GTGTGT). Primers that are "poor" by these criteria can cause artifacts that prevent amplification of the desired sequence. In some cases, these potential problems are difficult to avoid due to constraints imposed by the sequence of interest; in these cases, the use of longer primers (*i.e.* ≥ 24 bases) may solve specificity problems.

The primer should be long enough to have a reasonable melting temperature (*i.e.* a melting temperature of 55-70°C). Melting temperature depends on a number of variables. In most cases, an approximation [∑(4°C for each G or C) + (2°C for each A or T)] will yield a value close enough to design the PCR experiment.

PCR also allows the generation of mutations at the ends of the fragment, because the primer does not need to be an exact match to the template DNA (it needs to be a sufficiently good match to allow primer binding, but it does not need to be a perfect match). In most cases, the introduction of mismatches at the 5´ end of the primer has little effect. However, mismatches at the 3´ end of the primer may prevent synthesis of the new strand.

The mutations inserted by PCR are frequently used to generate restriction sites to simplify cloning of the PCR fragment. Although PCR can also be used to generate mutations within coding sequences, this is somewhat more complex, because the PCR primers only affect the sequence at the ends of the PCR fragments. However, modified forms of PCR are quite useful for site-directed mutagenesis experiments.

In order to amplify a sequence, we need a source of the actual coding sequence, and we need to know the sequence of the ends to allow the design of primers. If the PCR is intended to generate a coding sequence, genomic DNA from prokaryotes can be used as a template. For higher eukaryotes, however, cDNA must be used as a template to allow contiguous coding sequences to be amplified.

PCR requires the use of a DNA polymerase to make the copies of the DNA sequence used as a template. As noted in the figure above, the PCR method involves heating the sample to ~94°C to separate the chains of the double-stranded DNA. In addition, while the oligonucleotide binding and polymerization reactions can occur at a range of temperatures, oligonucleotide binding is much more specific (*i.e.* it is more likely that the oligonucleotide will bind the correct sequence) at higher temperatures. To prevent problems with low temperature incubations and problems due to denaturation of the polymerase, most PCR experiments employ thermostable DNA polymerases. The DNA polymerase most commonly used for PCR is derived from the bacterium *Thermus aquaticus*. *T. aquaticus* prefers to live at a temperature of about 70°C, and therefore its proteins (including its DNA polymerases) are stable at elevated temperatures.

Although the *Taq* polymerase is highly thermostable, it does begin to denature at temperatures above 90°C. Its half-life decreases rapidly with increasing temperature. For this reason, melting times of greater than 1 minute for the chain

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reaction cycles should be avoided. If the thermal cycler melting temperature drifts above 95°C, the enzyme may be inactivated prior to completion of the program.

The *Taq* polymerase has a primer extension rate of 60-100 bases/second under optimum conditions; thus it may be advantageous to use short (1-10 second) extension times, particularly for short products (*i.e.* below 500 bp) to decrease formation of non-specific products. However, for longer fragments (greater than 1000 bases), optimum primer extension rates are rarely achieved, and longer extension times should be used.

Although the *Taq* polymerase is a popular enzyme due to its ability to catalyze primer extension under a wide variety of conditions, it has some drawbacks. Its worst drawback is its relative lack of fidelity; it has a significant error rate (1 in 10^3 to 1 in 10^5 depending on the sequence), and lacks proofreading functions. As a result, many experiments employ thermostable DNA polymerases that have lower probabilities of misincorporation. The use of other polymerases may require minor modifications to the PCR procedure described below.

PCR conditions, and especially annealing temperature, must be chosen empirically to optimize PCR product formation. Some primer-template combinations work under a wide variety of conditions; others result in product only in a narrow range of conditions. For some reactions the annealing temperature is a critical parameter, with no specific product formed except in a narrow optimum range. It is usually best to begin testing conditions with an annealing temperature of about 55°C, because the use of annealing temperatures above 50°C often prevents certain types of mismatch artifacts.

The temperature profile shown below is a typical one that works for many different primers and templates:

PCR procedure:

Mix reagents in a PCR tube:
16 μl of mixture of 1.25 mM dNTP
10 μl 10x PCR buffer
5 μl 20 μM 5´ primer
5 μl 20 μM 3´ primer
1 μl of DNA template mixture
62.5 μl deionized water
0.5 μl *Taq* polymerase
Place the PCR tube in the PCR machine.

PCR Temperature Profile

Temp.	Time	Function
94°C	0.5 minutes	Denature DNA
55°C	0.5 minutes	Primer Annealing
72°C	0.75 minutes	Primer Extension

Run the PCR program as shown above (with appropriate modifications if necessary for the specific polymerase, primers, and template being used in the experiment).

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