7.1: Polymerase chain reaction overview

The polymerase chain reaction (PCR) revolutionized molecular biology. With PCR, researchers had a tool for amplifying DNA sequences of interest from extremely small amounts of a DNA template. Indeed, billions of copies can be synthesized from a single DNA molecule in a typical PCR reaction. The development of PCR grew out of research on DNA polymerases and the discovery of thermostable DNA polymerases able to withstand extended heat treatments that denature most proteins (Sakai et al., 1988). Today, PCR is a standard technique that is widely used to analyze DNA molecules and to construct novel recombinant molecules.

Thermostable DNA polymerases are central to PCR. The first description of PCR used a DNA polymerase from E. coli, which denatured and had to be replaced after each round of DNA synthesis (Sakai et al., 1985). The procedure was much-improved by replacing the E. coli polymerase with a DNA polymerase from Thermus aquaticus, a bacterium that thrives in thermal springs at Yellowstone National Park. The T. aquaticus DNA polymerase, or Taq polymerase, functions best at temperatures of 70-75 °C and can withstand prolonged (but not indefinite) incubation at temperatures above 90 °C without denaturation. Within a few years, the Taq polymerase had been cloned and overexpressed in E. coli, greatly expanding its availability. Today, the selection of polymerases available for PCR has increased dramatically, as new DNA polymerases have been identified in other thermophilic organisms and genetic modifications have been introduced into Taq polymerase to improve its properties.

PCR involves multiple rounds of DNA synthesis from both ends of the DNA segment that is being amplified. Recall what happens during DNA synthesis: a single-stranded oligonucleotide primer binds to a complementary sequence in DNA. This double-stranded region provides an anchor for DNA polymerase, which extends the primer, ALWAYS traveling in the 5’ to 3’ direction. Investigators control the start sites for DNA replication by supplying oligonucleotides to serve as primers for the reaction (shown below for Your favorite gene Yfg). To design PCR primers, investigators need accurate sequence information for
the primer binding sites in the target DNA. (Note: Sequence information is not needed for the entire sequence that will be amplified. PCR is often used to identify sequences that occur between two known primer binding sites.) Two primers are required for PCR. One primer binds to each strand of the DNA helix.

PCR begins with a denaturation period of several minutes, during which the reaction mixture is incubated at a temperature high enough to break the hydrogen bonds that hold the two strands of the DNA helix together. Effective denaturation is critical, because DNA polymerase requires single-stranded DNA as a template. The initial denaturation segment is longer than subsequent denaturation steps, because biological templates for PCR, such as genomic DNA, are often long, complex molecules held together by many hydrogen bonds. In subsequent PCR cycles, the (shorter) products of previous cycles become the predominant templates.

Following the initial denaturation, PCR involves a series of 30-35 cycles with three segments, performed at different temperatures. PCR reactions are incubated in thermocyclers that rapidly adjust the temperature of a metal reaction block. A typical cycle includes:

- a denaturation step - commonly 94°C
- a primer annealing step - commonly 55°C
- an extension step - commonly 72°C
PCR reactions include multiple cycles of denaturation, annealing and extension.

Each cycle of PCR includes three different temperatures. In the denaturation step, the hydrogen bonds holding DNA helices together are broken. In the annealing step, oligonucleotide primers bind to single-stranded template molecules, providing starting points for processive DNA polymerases that extend the primer sequence. DNA polymerases become more active at the extension temperature, which is closer to their optimal temperature. Investigators adapt the temperatures and timing of the steps above to accommodate different primers, templates and DNA polymerases.

PCR products of the intended size accumulate exponentially

PCR is indeed a chain reaction, since the DNA sequence of interest roughly doubles with each cycle. In ten cycles, a sequence will be amplified ~1000 fold \((2^{10}=1024)\). In twenty cycles, a sequence will be amplified ~million fold. In thirty cycles, a sequence can be theoretically amplified ~billion fold. PCR reactions in the lab typically involve 30-35 cycles of denaturation, annealing and extension. To understand PCR, it's important to focus on the first few cycles. PCR products of the intended size first appear in the second cycle. Exponential amplification of the intended PCR product begins in the third cycle.

During the first cycle, the thermostable DNA polymerases synthesize DNA, extending the 3’ ends of the primers. DNA polymerases are processive enzymes that will continue to synthesize DNA until they literally fall off the DNA. Consequently, the complementary DNA molecules synthesized in the first cycle have a wide variety of lengths. Each of the products, however, has defined starting position, since it begins with the primer sequence. These “anchored” sequences will become templates for DNA synthesis in the next cycle, when PCR products of the intended length first appear. The starting template for PCR will continue to be copied in each subsequent cycle of PCR, yielding two new “anchored” products with each cycle. Because the lengths of the “anchored” products are quite variable, however, they will not be detectable in the final products of the PCR reaction.

![First cycle of PCR](https://bio.libretexts.org/Bookshelves/Cell_and_Molecular_Biology/Book%3A_Investigations_in_Molecular_Cell_Biology_(O%C2%B2C…
Updated: Mon, 25 May 2020 13:37:34 GMT
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DNA strands of the intended length first appear during the second cycle. Replication from the “anchored” fragments generates PCR products of the intended length. The number of these defined length fragments will double in each new cycle and quickly become the predominant product in the reaction.
Most PCR protocols involve 30-35 cycles of amplification. In the last few cycles, the desired PCR products are no longer accumulating exponentially for several reasons. As in any enzymatic reaction, PCR substrates have become depleted and the repeated rounds of incubation at 94°C have begun to denature Taq polymerase.

Primer annealing is critical to specificity in PCR

Good primer design is critical to the success of PCR. PCR works best when the primers are highly specific for the target sequence in the template DNA. Mispriming occurs when primers bind to sequences that are only partially complementary, causing DNA polymerase to copy the wrong DNA sequences. Fortunately, investigators are usually able to adjust experimental parameters to maximize the probability that primers will hybridize with the correct targets.

PCR primers are typically synthetic oligonucleotides between 18 and 25 bases long. When designing a primer, researchers consider its T_m, the temperature at which half of the hybrids formed between the primer and the template will melt. In general, the thermal stability of a hybrid increases with the length of the primer and its GC content. (Recall that a GC-base pair is stabilized by three H-bonds, compared to two for an AT pair.) The following formula provides a rough estimate of the T_m of oligonucleotide hybrids. In this formula, n refers to the number of nucleotides, and the concentration of monovalent cations is expressed in molar (M) units.

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T_m = 81.5 \, ^\circ C + 16.6 \cdot \log_{10}(K^+ + Na^+) + 0.41 \cdot (G + C) - (675/n)
\]

When possible, researchers design primers that are similar in length and have a 40-60% GC composition. The sequences are designed so that the T_mS of the primer-DNA hybrids are within a few degrees of the annealing temperature. Adjusting the T_mS of the primers to be close to the annealing temperature favors specific hybrids over less specific hybrids that may contain a few mismatched bases. A hybrid formed between a primer and a non-target sequence with even one mismatched base has a T_m that is lower than that of the fully hydrogen-bonded hybrid. If DNA polymerase extends the mismatched primer, incorrect PCR products will be generated. When mispriming appears to be a problem in a PCR reaction, investigators have several options to increase the yield of the desired product. They can increase the length and/or GC content of the primers, alter the salt concentrations (results may be hard to predict) or increase the annealing temperatures.

When designing a PCR reaction, investigators also consider the nature of the template DNA. A variety of DNA templates can be used for PCR. Depending on the purpose of the experiment, investigators could choose to use genomic DNA, a plasmid or a cDNA (complementary DNA generated by a reverse transcriptase from mRNA). PCR can also be done with much cruder preparations of DNA, such as a bacterial or yeast colony. The more complex the template (its length in bp), the greater the probability that it will contain another sequence that is very similar to a primer sequence. For example, the haploid yeast genome is 12 Mbp long and contains only one copy of each MET gene. The probability that a non-target sequence in the yeast genome is similar...
enough to a 25-nucleotide *MET* primer to cause mispriming is reasonably good. Furthermore, these sequences with small mismatches may outnumber the target sequence. With complex templates such as genomic DNA, therefore, investigators can sometimes reduce the impact of mismatched hybrids by decreasing the amount of template DNA in the reaction. (Using too much template is the most common error in yeast colony PCR.)

The components of a PCR reaction are simple, consisting of the DNA template, primers, dNTPs, a buffer containing MgCl₂ (polymerases use dNTPs complexed with Mg²⁺), and the thermostable polymerase. **For our experiments, we will be using a master mix that contains all of the components except the template DNA and the primers.** The use of a master mix ensures that all reactions have identical reagents and it also reduces the number of transfers requiring micropipettes. The smaller number of transfers is particularly advantageous, because it reduces the opportunities for cross-contamination of reagents. PCR is an exquisitely sensitive procedure. Some researchers even use special barrier tips for their micropipettes, which contain filters that prevent samples from reaching the barrel of the micropipettes.