The Polymerase Chain Reaction

One of the most important advances in molecular biology, has been the polymerase chain reaction.

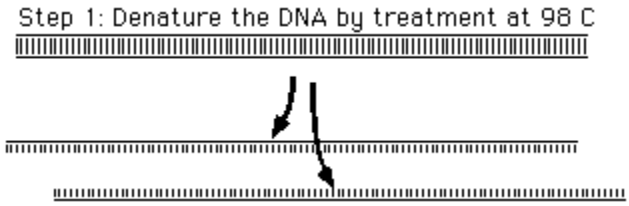
In the polymerase chain reaction, a DNA template is repetitively:

- denatured into single stranded molecules,
- annealed to specific oligonucleotide primers (one specific primer per strand),

copied with DNA polymerase to extend the primers to the end of the DNA strand

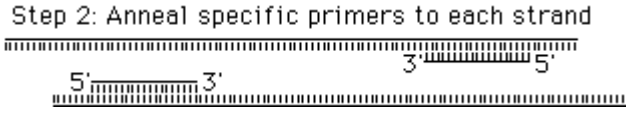
Amplification

Step 1: Denaturation
(95°C-98°C)



Why must the DNA be denatured into single strands? Because without separation of strands, you would not be able to anneal (i.e. hybridize) specific primers in the next step.

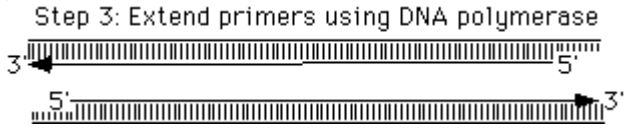
Step 2: Annealing
(45°C-65°C)



Primers in excess

The annealing reaction is very efficient because the primers are "in excess" in the reaction. In a typical PCR reaction, 10,000 molecules of a template may be used, which is 1.6 x 10^{-20} moles (0.016 attomoles). On the other hand, 5 picomoles of each primer may be used (5 x 10^{-12} moles) -- that is a 3 x 10^{8} fold excess.

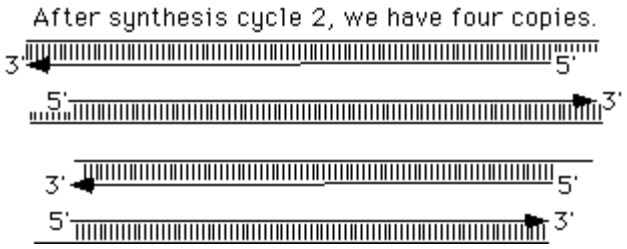
Step 3: Extension
(65°C-75°C)



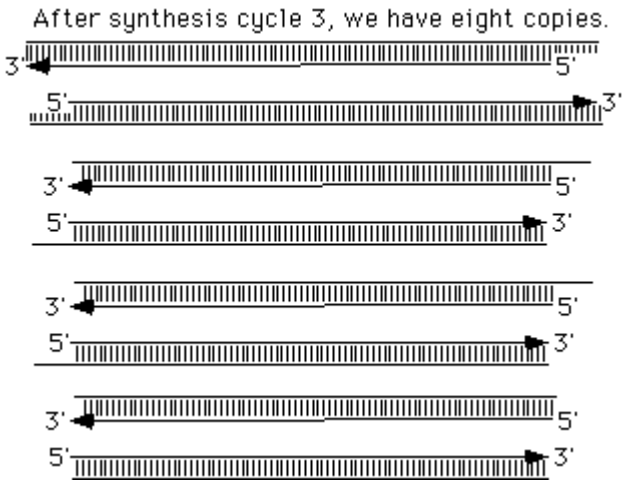
Temperature controls annealing rate

The rate of annealing is controlled by adjusting the temperature of the solution. At 55 C under most PCR salt conditions, typical primers of 18 nt. in length efficiently hydrogen bond to a DNA template. Adjustments in the protocol are made to account for the G/C vs. A/T richness of the primer and the overall length. There are many programs or Web sites at which one may calculate the Tm (melting temperatures) based on sequence and salt condition.

You can see, from a comparison of the figures for step 1 and step 3, that we now have two double-stranded DNA copies of the sequences between the specific primers. By denaturing these two copies and repeating the annealing and synthesis steps, we can obtain four copies.



Repeat!!! Now if we repeat the process again, we can obtain eight copies.



Note that the sequence between the two primers is being copied or "amplified" exponentially, whereas the original template is not.

Many of these copies have 3' overhanging ends, because the primer sequences are only extended in one direction (synthesis is only 5' to 3'). These longer versions are generated only from the original template, and not from the copies; as a result they are not generated at an exponential rate. On the other hand, the shorter versions (as in the bottom two molecules in the figure above) contain copies of the DNA between the two primer sequences, and are "amplified" at an exponential rate (1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, and so forth). If things worked perfectly, we could obtain approximately a 1000-fold amplification for every ten cycles of synthesis!

An overview of what is needed:

A pair of short oligonucleotide primers specific for a DNA sequence, with the ability to hybridize to the opposite strands of that molecule (3' ends pointing "towards" each other):

- A DNA template.
- A thermostable DNA polymerase (such as Taq or Pfu polymerase), and all four dNTP substrates (meaning dATP, dGTP, dCTP, dTTP)
- A machine that can change the incubation temperature of the reaction tube automatically, cycling between approximately 98 C (for denaturation), 55 C (for oligonucleotide annealing), and 72 C (for synthesis).

Where does the thermostable polymerase come from?

These days, the thermostable polymerases are commercial products, but not too long ago they were only found in hot springs (such as this one at Yellowstone National Park).

The temperature changes:

When you program the thermocycler, you specify a series of temperatures and times, such as:

Temperature	**Time**
98 C	30 seconds
55 C	30 seconds
72 C	60 seconds

...and specify the number of times this series should be repeated (for example, 35 times). Multiple program segments may be linked together, as in the following example:

Temperature	**Time**
program segment 1, do 1 time:	
98 C	5 minutes
program segment 2, do 35 times:	
98 C	30 seconds
55 C	30 seconds
72 C	60 seconds
program segment 3, do 1 time:	
72 C	10 minutes
program segment 4, do 1 time:	
4 C	999 minutes

What was the purpose of each of these segments?

Program segment #1: To denature the template fully, prior to the first synthetic step. If this step is excessive, there can be damage to the enzyme or template, reducing the efficiency. If too short, the template is not available for synthesis.

Program segment #2: To amplify the DNA fragment, with the following steps taken:

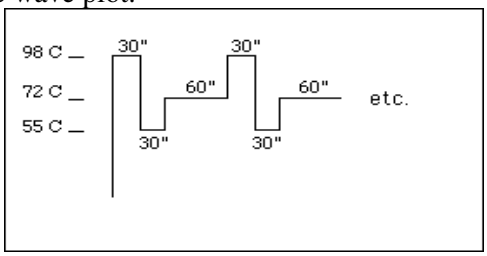
- Denature DNA at 96 C
- Anneal oligonucleotides at 55 C
- Synthesize DNA at 72 C

The individual temperatures (96, 55, 72) may be optimized for each reaction, however the thermostable polymerases generally work well at 72-74 C, and typical oligonucleotides anneal well at 45-65 C.

Program segment #3: Finish synthesis of any partially completed fragments.

Program segment #4: Cool samples while waiting for researcher to finish nap.

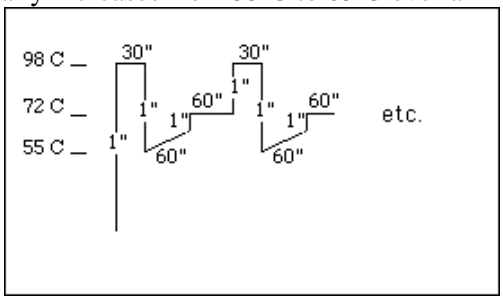
In this type of program, the temperature changes are as rapid as the machine can manage, usually taking 30 seconds to a minute to complete. Some advanced machines can change the temperature between these steps in just seconds, and these speed up the PCR process considerably. This type of temperature profile could be represented by a square wave plot.



There are times when you don't want the temperature changes to be rapid, and here's an example: Suppose we are trying to work out the conditions for a polymerase chain reaction using two degenerate oligonucleotides that are approximately 1000-fold degenerate. We would like to use that handy web site to determine the Tm, so we would know what annealing temperature to program into the machine, but we don't actually know which of the 1000 versions of each oligonucleotide will be an exact match to the target sequence. What we would really like to do is to introduce some flexibility into the temperature cycle, so that every potential oligonucleotide has a fair chance of annealing to the target.

What do we do?

Answer: We program the temperature cycler so that it gradually changes the annealing temperature, thereby exposing the reaction to a range of temperatures. In this kind of program (characteristic of File #3 in the Perkin Elmer 480 instrument), the timing at each temperature and between each temperature are specified. Rapid changes can be programmed by setting a "between temperature" time of only one second (it obviously takes longer than that to change temperatures, so the machine just does its best). In this example, the temperature gradually increases from 55 C to 65 C over a 1 minute period.



Otherwise, you will notice how similar it is to the square-wave version described before.
If you' ve got your MasterCard ready, here are some of the models (past and present) from the Perkin Elmer showroom:

The vapor pressure problem

With the Perkin Elmer 480, it is often recommended that you use some sort of mineral oil overlay on your sample, to keep it from condensing on the top of the tube (which is cooler than the bottom). The reason to worry about condensation is that it removes water from your reaction mixture by distillation, and makes the salts (and other components) more concentrated. On a more modern instrument such as the Perkin Elmer 2400, there is a heated cover that keeps the top of the tube warm, and prevents it from acting as a site of condensation. Nonetheless, loss of solution volume can be troubling and a source of irreproducibility. ***Actual yield is less than the theoretical maximum***

You may recall our discussion about the maximal theoretical yield, which is to double the amount of product every cycle. In practice you do not achieve that level of synthesis.
Here is an example of synthesis specifications from Perkin Elmer Corp.:

> *Using the DNA Thermal Cycler 480 and the GeneAmp® PCR Reagent Kit, an amplification yield of at least 100,000 fold of the Lambda Control DNA target can be achieved with: 0.2 µM each of the Lambda Control Primers. 0.1 ng of Lambda Control DNA target. 100 µL reaction volume with a 50 µL mineral oil overlay in a 0.5 mL GeneAmp® Thin-Walled Reaction Tube. >25 thermal cycles: 94 degrees C for 1 minute and 68 degrees C for 2 minutes.*

An amplification yield of 100,000x after 25 cycles would mean at each cycle 1 template would yield 1.58 templates for the next round of synthesis.

> How was this calculated? If c is the number of copies made per round of synthesis then
> $c^{25} = 100{,}000 = 10^5$
> so $c^5 = 10$
> and so $5(\log c) = \log 10 = 1$
> so $\log c = 0.2$ and $c = 1.58$ (approximately)
>
> (Or you could calculate the 25th root of 100,000 on a calculator, if you prefer.)

If we obtain 1.58 copies instead of the theoretical maximum of 2 copies, then the efficiency of the reaction could be said to be 79% (because 1.58/2.00 = 0.79).

One reason this calculation is important, is that a slight loss of efficiency is magnified through the amplification. A reaction may appear to have not worked if the efficiency drops (in each cycle) by just a few percent. Optimization is critically important in the polymerase chain reaction.

Here are some comments, abstracted from the Pfu Turbo DNA polymerase manual from Stratagene Co.

Extension time is one of the most critical parameters affecting the yield of PCR product obtained. For optimal yield with minimal smearing using pfuTurbo DNA polymerase, use an extension time of 1.0 minute/kb for vector targets up to 10 kb and genomic targets up to 6 kb. When amplifying vecotor targets between 10 and 15 kb or genomic targets between 6 and 10 kb in length, use an extension time of 2.0 minutes/kb.

The most successful PCR results are achieved when the amplification reaction is performed using purified primers and templates that are essentially free of extraneous salts. Gel-purified primers, generally > 18 nucleotides in length, are strongly recommended. Additionally, an adequate concentration of primers and template should be used to ensure a good yield of desired PCR products. When DNA of known concentration is available, amounts of 50-1000 ng of DNA template/100 ul reaction are typically used for amplifications of single-copy chromosomal targets. Stratagene suggests using primers at a final concentration of 0.1-0.5 uM.

Specificity problem

The appropriate annealing temperature can be calculated from the base sequence and length, noting that longer oligonucleotides can form more hydrogen bonds with a target and therefore have a higher annealing temperature. Similarly, the fraction of G or C nucleotides in an oligonucleotide affects the annealing temperature because GC base pairs form three H bonds and AT base pairs form only two. If there is any degeneracy or mis-match between the oligonucleotide and the target, the annealing temperature will be lower.

A typical annealing temperature one might use is 50 to 60 degrees Celsius. There is a problem with using annealing temperatures lower than that, because nonspecific products may be amplified. These are side reactions in which an oligonucleotide may form just a few Watson Crick base pairs with a template, and are usually unwanted. Here is an example:

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GGATAGGACCTAGGAGGACCAGGAGATCCCGCCTACCGAAGGACG-3'
                              |||||| || ||
    synthesis  <---- CTAGGGATTATAGCACATT-5'
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If another starting site is found further to the left, and pointing in the opposite direction (with either this oligo or its partner in the reaction), then a nonspecific product may be made in the reaction. The ability of that side reaction to compete with the specific reaction will depend on the length of the product. Smaller products are synthesized faster, and are therefore more competitive. Note that even though this side reaction was only initiated with a few H bonds, subsequent reactions in the tube will fused to the oligonucleotide. That means they will be able to anneal "end to end" with the oligonucleotide.

What do you get if you have numerous nonspecific side reactions?

Answer: When you run your reaction products on a gel, you will get a smear instead of a band.

How can you solve this problem?

Answer: There are several things you can try.

- Raise your annealing temperature
- Perform a "hot start", meaning that the reaction is not initiated until the tube is at a temperature above approximately 50 C. Sometimes this is done by leaving out one of the reaction components (such as the Taq polymerase) until a certain temperature is reached. There are also waxes you can

buy to keep two halves of a reaction mix apart (until the wax melts that is). Why is this important? Because it prevents low-temperature annealing and synthesis when your PCR machine is just warming up the tubes for the first cycle. There are also polymerases commercially available that are complexed with an inactivating antibody - when the temperature rises during the initial denaturation, the antibody is denatured (it is not thermostable) and the polymerase is free to do its job.

- Design new oligonucleotides that have fewer matches with nonspecific sequences