

POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) can be very frustrating. Sometimes it works and sometimes it doesn't even when you think you've done everything the same way. The basis of the reaction is very simple-- utilizing at least two specific primers, a DNA template, dNTPs and a thermal stable polymerase in a buffered solution --a specific fragment of DNA is amplified. The diagram on the following page illustrates how a desired DNA fragment is specifically amplified.

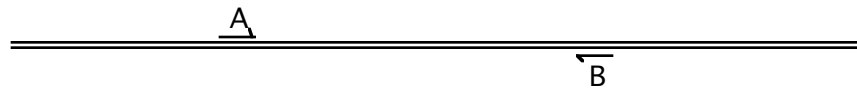
One of the main complaints in PCR is that it is unreliable since the results can be inconsistent from attempt to attempt. However, if all (and I do mean all) of the conditions are the same then you will get the same results from every attempt.

To avoid reproducibility problems it may help to follow the few helpful hints outlined below.

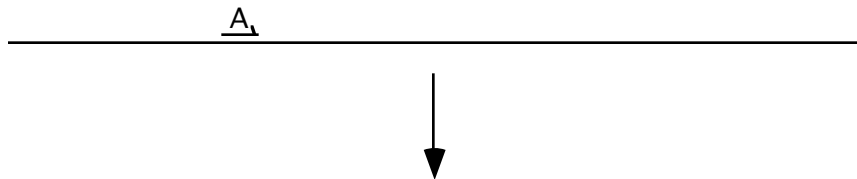
1. Keep your reagents at -20°C , thaw them in r.t. water and vortex them prior to using.
2. Always set up your reactions on ice and keep all your reagents on ice after thawing them.
3. Set up your reactions in the following addition order: 1. dH_2O , 2. buffer, 3. template DNA, 4. primers, 5. dNTPs and 6. thermal stable polymerase. The water is added to your reaction tube first since it is usually the largest volume and by adding it first allows the water to cool to $5-6^{\circ}\text{C}$ prior to the addition of the polymerase. The nucleic acids are added in order of stability.
4. Reactions usually work better when the total volume per reaction is $<20\mu\text{l}$. One can use larger volumes but the overall efficiency of the reaction may be less. If you need to amplify more DNA than which can be produced in a $20\mu\text{l}$ reaction then mix up the volume which you require and then split it into $20\mu\text{l}$ aliquots. This effect is caused by uneven heating/cooling in larger volumes relative to the smaller volumes where one has a better surface area to volume ratio.

Basics of PCR:

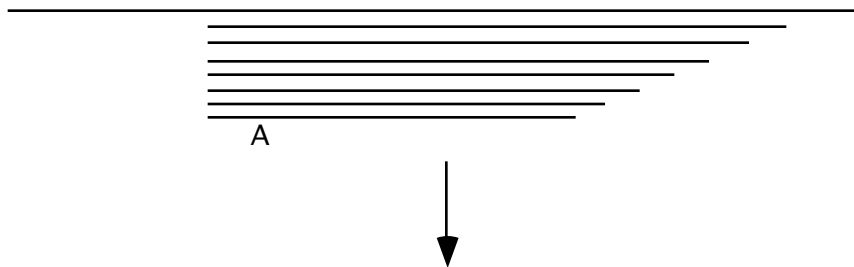
Primer A & B define the DNA fragment to be amplified



In the annealing phase of the first round of the reaction, following the denaturation phase, the primer hybridizes to the template strand (for now only concern yourself with primer A).



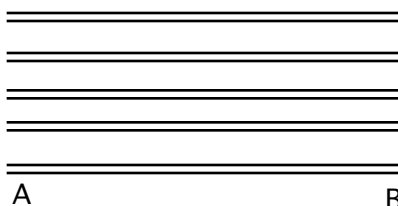
During the polymerization phase of the first round DNA fragments of random length are produced (remember, however, the 5' end is not random since it is defined by the primer). Similar fragments are also formed utilizing primer B, however, they are of the opposite sense.



In the second round we shall consider what can happen to primer B. Primer B can now not only hybridize to the original template DNA but it can also hybridize to the DNA fragments amplified from primer A. DNA fragments amplified from primers annealed to the template result in random length fragments (same as DNA fragments, however, amplified from primers annealed to fragments polymerized utilizing primer A will now not only have a defined 5' end but will also have a defined 3' end (these fragments are illustrated below).



In the third round we shall now switch back to primer A. Primer A can now hybridize to the original template fragments polymerized from primer B with either random or defined 3' ends. The primer A's which hybridize to the primer B fragments that have defined 5' & 3' ends (as illustrated above) will now create double-stranded DNA fragments with a defined length as shown below.



Considerations for amplifying DNA using PCR:

1. The most critical step in PCR is actually the first step you do and that is primer selection. The best way to select a primer is to consider the following points:
 - A. The last three 3' nucleotides of both primers should not be able to hybridize to themselves or to the other primer. If this can occur then the primers will form primer dimers in solution, the polymerase will extend these dimers and essentially create new primers which will be non-specific.
 - B. Match the melting temperatures of the two primers. A simple rule for determining the T_m of a primer is the 2+4 rule or 2°C for every A or T nucleotide and 4°C for every G or C nucleotide. A good range of T_m to stay within is 40-55°C.
2. The typical parameters for a cycle in PCR are the following: 10sec.-1min. at 94°C, 30sec.-2min. at the primer annealing temp. and 1-3min. at 72°C. The length of time varies depending on the type of tube/titer plate you're using to do the PCR. Our lab currently uses thin walled titer plates which do not require long time periods for the denaturation (~30 sec. at 94°C) and annealing (30sec-1min) steps.
3. The number of cycles to be used in a single reaction varies according to the type of amplification you are doing. Typical PCR's use between 20-40 cycles. Remember that as you increase the number of cycles you increase the possibility of obtaining a mutation in your final PCR product.

Always remember to sequence your final PCR product (i.e. the clone).
4. A good initial step in amplifying a fragment of DNA is to vary the buffer conditions and hold all other components constant.

Initial concentrations for the other components are the following:
200µM dNTPs, 1µM oligo A, 1µM oligo B, 1ng template DNA/10µl of reaction volume (assuming template is 5kb in length)

A good buffer series to try is the following (all given concentrations are 10x):

 1. 100mM Tris pH8.4, 500mM KCl and 15mM MgCl₂
 2. 100mM Tris pH8.8, 470mM KCl and 27mM MgCl₂
 3. 100mM Tris pH8.4, 500mM KCl and 20mM MgCl₂
 4. 100mM Tris pH8.8, 500mM KCl, 60mM MgCl₂ and 10mM DTT
 5. 100mM Tris pH8.4, 500mM KCl and 60mM MgCl₂
 6. 1M Tris pH9.0, 400mM (NH₄)₂SO₄ and 30mM MgCl₂
5. Once one has established which buffer is best, the concentration of the other components can be varied in order to maximize the efficiency of the reaction. This step is usually not necessary for most PCR's, however, for sensitive amplifications it may be necessary.

