A Basic Polymerase Chain Reaction Protocol

Introduction

The polymerase chain reaction (PCR) is the cardinal laboratory technology of molecular biology. Arguably one of the most powerful laboratory techniques ever discovered, PCR combines the unique attributes of being very sensitive and specific with a great degree of flexibility. With the PCR it is possible to specifically address a particular DNA sequence and to amplify this sequence to extremely high copy numbers. Since its initial development in the early 1980's, dozens of variations in the basic theme of PCR have successfully been carried out. In fact, the very flexibility and application-specific variation of PCR make it seem like there are as many ways to do a PCR reaction as there are researchers doing them. Here, a basic, straight-forward PCR protocol is presented. Where appropriate, some of the choices for modifying this standard reaction that are routinely available to researchers are discussed.

Step 1: Choosing Target Substrates and PCR Primers

The choice of the target DNA is, of course, dictated by the specific experiment. However, one thing is common to all substrate DNAs and that is they must be as clean as possible and uncontaminated with other DNAs. Naturally, if the source material is an environmental sample such as water or soil, then the researcher must rely upon the specificity of the PCR primers to avoid amplification of the wrong thing.

Specificity in the choice of PCR primers should be an issue in any PCR amplification. The on-line IDT SciTools software OligoAnalyzer 3.0 and PrimerQuest are invaluable aids both in primer design and validation. PrimerQuest will assist in primer design and will permit the researcher to directly assess primer specificity via a direct BLAST search of the candidate sequences (see the Bioinformatics Tutorial). Taking candidate primer sequences into OligoAnalyzer will allow for each primer sequence to be assessed for the presence of secondary structures whether these are hairpins or homo- and hetero-dimers. Many of the basic analyses available in PrimerQuest and OligoAnalyzer are presented in the Polymerase Chain Reaction Tutorial.

Step 2: Setting Up the Reaction

Once you have chosen the appropriate substrate and your PCR primer sequences and you have them on hand, the basic reaction components are as follows:

Water
10x Reaction Buffer
$MgCl_2$

dNTPs
Forward Primer
Reverse Primer
Target DNA
Polymerase enzyme

The role that each of these components plays in a PCR reaction is discussed in the Tutorial entitled Polymerase Chain Reaction. One common choice available to the researcher is whether or not to use a Reaction Buffer that already contains the magnesium chloride. The vast majority of PCR reactions will work perfectly well at a 1.5 millimolar (mM) magnesium chloride concentration. For this reason PCR reaction buffers that do contain $MgCl_2$ are prepared so that the final concentration is 1.5mM in the 1:10 dilution. Occasionally, however, $MgCl_2$ final concentrations other than 1.5mM may be optimal. When this occurs it becomes necessary to use a reaction buffer that does not contain $MgCl_2$ and to add the $MgCl_2$ separately.

Step 3: Choosing the Reaction Conditions

The reaction conditions of a PCR amplification are composed of the total number of cycles to be run and the temperature and duration of each step in those cycles. The decision as to how many cycles to run is based upon the amount of DNA target material you start with as well as how many copies of the PCR product (amplicon) you want. In general, 25 to 35 cycles is the standard for a PCR reaction. This results in from approximately 34 million to 34 billion copies of the desired sequence using 25 cycles and 35 cycles respectively. Additional cycle numbers can be used if there is a small amount of target DNA available for the reaction. However, reactions in excess of 45 cycles are quite rare. Also, increasing the number of cycles for larger amounts of starting material is counter productive because the presence of very high concentrations of the PCR product is itself inhibitory [1].

Once the number of cycles is selected, it is necessary to choose the temperature and duration of each step in the cycles. The first step is the **DNA denaturation step** that renders all of the DNA in the reaction single stranded. This is routinely accomplished at 94^{o}C or 95^{o}C for 30 seconds. The second step is the **primer annealing step** during which the PCR primers find their complementary targets and attach themselves to those sequences. Here the choice of temperature is largely determined by the melting temperature (Tm) of the two PCR primers (see OligoAnalyzer 3.0 in IDT SciTools). Again, the usual duration is 30 seconds. Finally, the last step in a PCR cycle is the **polymerase extension step** during which the DNA polymerase is producing a complementary copy of the target DNA strand starting from the PCR primer sequence (thus the term primer). The usual temperature of this step is 72^{o}C, considered to be a good optimum temperature for thermal-stable polymerases. A common rule of thumb for the duration of this step has been 30 seconds for every 500 bases in the PCR product. However, with the increasing quality of commercially available polymerase enzymes and the associated reaction components, this time can be significantly shortened but should be done in a

systematic manner since the optimal extension time can be polymerase and sequence specific. In addition to these cycling conditions, it is often desirable to place a single denaturation step of three to five minutes at 94°C or 95°C at the beginning of the reaction and a final extension step of a few minutes at 72°C. A convenient shorthand way of representing a complete set of reaction conditions is:

5 min. 94°C; 35 x (30 sec 94 °C, 45 sec 60 °C, 2 min 72 °C); 7 min 72 °C

which means an initial denaturing step of five minutes at 94°C followed by 35 cycles of 94°C for 30 seconds, 60°C for 45 seconds and 72°C for two minutes and then a final extension at 72°C for seven minutes.

Step 4: Validating the Reaction

Once your PCR reaction has run, there are two ways of determining success or failure. The first is to simply take some of the final reaction and run it out on an agarose gel with an appropriate molecular weight marker to make sure that the reaction was successful and if the amplified product is the expected size relative to the maker (see Gel Electrophoresis Tutorial).

The ultimate validation of a PCR reaction is to directly sequence the amplicon. This is often a choice that is not readily available since not everyone has access to a DNA sequencer nor will they have either the time or the funds to carry out such an analysis. One way to indirectly assess the sequence of an amplicon, however, is to carry out restriction enzyme digests on it. Given the vanishingly low likelihood that two well chosen primers will amplify an incorrect amplicon that matches the expected size, it is even more unlikely that an incorrect amplicon will give an expected pattern of restriction fragments (see Restriction Endonucleases Tutorial).

References and Resources

1. Kainz P. (2000) The PCR plateau phase- towards an understanding of its limitations. Biochem Biophys Acta, 1494: 23–27.

For additional information, see the following resources for exploring the polymerase chain reaction and its variants.

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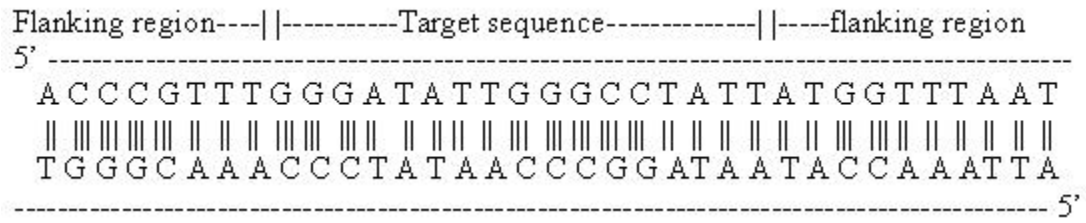
POLYMERASE CHAIN REACTION (PCR)

PCR stands for the Polymerase Chain Reaction and was developed in 1987 by Kary Mullis (which won him a Nobel Prize) and associates. With this technique it is possible to make virtually unlimited copies of a single DNA molecule even though it is initially present in a mixture containing many different DNA molecules. It is used to amplify a specific DNA (target) sequence lying between known positions (flanks) on a double-stranded (ds) DNA molecule. The polymerase chain reaction can be used to amplify both double and single stranded DNA.

In order to perform PCR, one must know at least a portion of the sequence of the target DNA molecule that has to be copied. Generally, PCR amplifies small DNA targets 100-1000 base pairs (bp) long. It is technically difficult to amplify targets >5000 bp long. A pair of single stranded oligonucleotide primers, which have DNA sequences complementary to the flanking regions of the target sequence, must be synthesized. The primers are complementary to either end of the target sequence but lie on opposite strands. The primers are usually 20-30 nucleotides long and bind to complementary flanking region at 3' end.

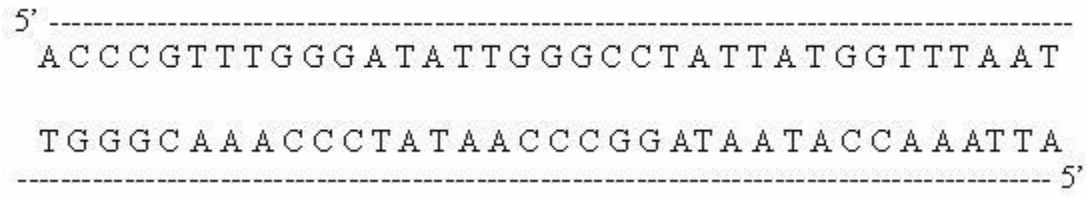


Requirements:

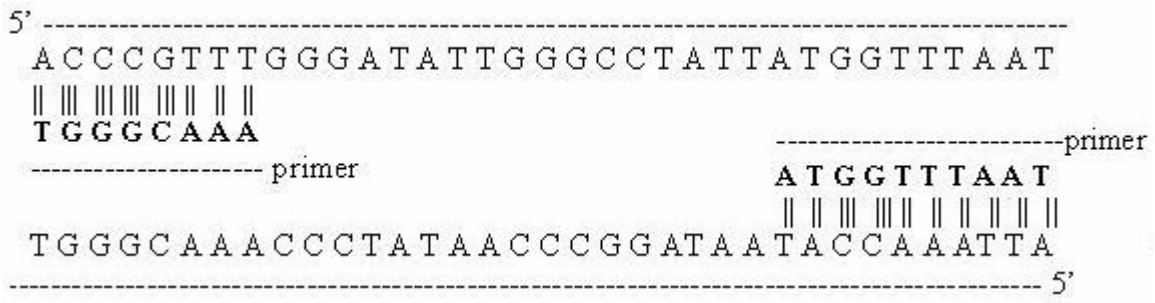
- Thermal cycler (thermocycler)
- PCR amplification mix typically containing:
- Sample dsDNA with a target sequence
- Thermostable DNA polymerase
- Two oligonucleotide primers
- Deoxynucleotide triphosphates (dNTPs)
- Reaction buffer containing magnesium ions and other components

Procedure:

1. The DNA molecule carrying a target sequence is denatured by heat at 90-95^{o}C for 20 seconds. The two strands separate due to breakage of the hydrogen bonds holding them together. Oligonucleotide primers are added.



2. A reaction mixture containing all four deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP) and a thermostable DNA polymerase is added. A DNA polymerase (Taq) that is not denatured by the high temperature needed to separate the DNA strands is used. It is usually sourced from *Thermus aquaticus*, a bacterium isolated from hot springs.
3. The mixture is allowed to cool to a lower temperature (50-65^{o}C). Each strand of DNA molecule becomes annealed with an oligonucleotide primer complementary to either end of the target sequence. Primer annealing takes 20 seconds.



4. The temperature is raised to 60-75^{o}C and primers are extended by the action of DNA polymerase for 30 seconds. The polymerase synthesizes complementary sequence the 5' to 3' direction away from each of the primers. If the template contains an A nucleotide, the enzyme adds on a T nucleotide to the primer. If the template contains a G, it adds a C to the new chain. Polymerization continues until each newly synthesized strand has proceeded far enough to contain the site recognized by the other primer. At this point there would be exactly two copies of the target DNA sequence.

5. The mixture is heated again at 90-95^{o}C to denature the molecules and separate the strands and the cycle repeated. Each new strand then acts as a template for the next cycle of synthesis. Thus amplification proceeds at an exponential (logarithmic) rate, i.e. amount of DNA produced doubles at each cycle. The amplified product at the end of PCR is called amplicon.

A typical thermal cycle might be as follows:

- Heat denaturation at 94^{o}C for 20 seconds
- Primer annealing at 55^{o}C for 20 seconds
- Primer extension at 72^{o}C for 30 seconds

Average time for each cycle is approximately 4-5 minutes, considering the fact that heating and cooling between each stage also have to be considered.

Initially these steps at three different temperatures were carried out in separate water baths but nowadays a thermal cycler is used (a machine that automatically changes the temperature at the correct time for each of the stages and can be programmed to carry out a set number of cycles). After the introduction of thermocycler, each cycle of replication can be completed in less than 5 minutes. After 30 cycles, a single molecule of DNA is amplified into more than a billion copies (2^{30} = 1.02 x 10^{9}).

Post amplification detection: Following PCR, the amplification product can be detected using gel electrophoresis followed by ethidium bromide staining and visualization with uv transillumination. Visualization of a band containing DNA fragments of a particular size can indicate the presence of the target sequence in the original DNA sample. Absence of a band may indicate that the target sequence was not present in the original DNA sample. Confirmation of the amplicons can be made by southern blotting using specific probes.

Modifications and different types of PCR are:

Nested PCR, Multiplex PCR, RT-PCR, Touchdown PCR, Arbitrarily Primed PCR, Inverse PCR, Allele Specific PCR, Asymmetric PCR, "Hot Start" PCR, Core Sample PCR, Degenerate PCR and PCR-Elisa.

Applications of PCR:

- Amplification of small amounts of DNA for further analysis by DNA fingerprinting.
- The analysis of ancient DNA from fossils.
- Mapping the human (and other species) genome.
- The isolation of a particular gene of interest from a tissue sample.
- Generation of probes: large amount of probes can be synthesized by this technique.
- Production of DNA for sequencing: Target DNA in clone is amplified using appropriate primers and then its sequence determined. Helpful in conditions where amount of DNA is small.
- Analysis of mutations: Deletions and insertions in a gene can be detected by differences in size of amplified product.
- Diagnosis of monogenic diseases (single gene disorders): For pre-natal diagnosis, PCR is used to amplify DNA from foetal cells obtained from amniotic fluid. PCR has also proved very important in carrier testing.
- Detection of microorganisms: Especially of organisms and viruses that are difficult to culture or take long time to culture or dangerous to culture.
- The PCR has even made it possible to analyze DNA from microscope slides of tissue preserved years before.
- Detection of microbial genes responsible for some aspect of pathogenesis or antibiotic resistance.
- Crucial forensic evidence may often be present in very small quantities, e.g. one human hair, body fluid stain (blood, saliva, semen). PCR can generate sufficient DNA from a single cell.

Limitations of PCR: PCR is an extremely sensitive technique but is prone to contamination from extraneous DNA, leading to false positive results. Another potential problem is due to cross-contamination between samples. It is for this reason that sample preparation, running PCR and post-amplification detection must be carried out in separate rooms. Concentration of Mg is very crucial as low Mg^{2+} leads to low yields (or no yield) and high Mg^{2+} leads to accumulation of nonspecific products. Non-specific binding of primers and primer-primer dimmer formation are other possible reasons for unexpected results. Reagents and equipments are costly, hence can't be afforded by small laboratories.

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