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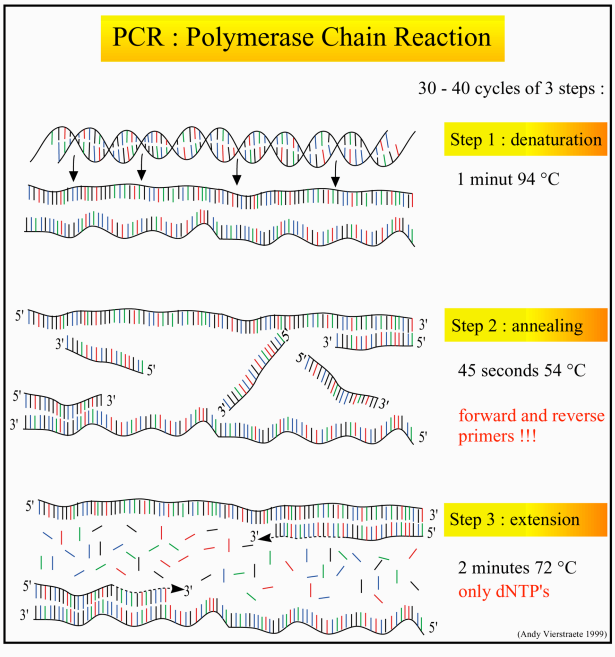
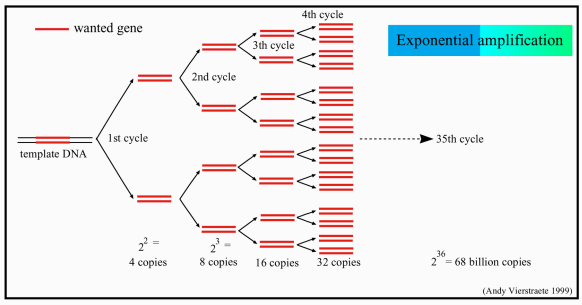
Polymerase Chain Reaction (PCR)

Background information

The polymerase chain reaction (PCR) is an enzymatic process that allows for the detection of specific genes within an environmental DNA sample. PCR utilizes short, user defined DNA sequences called oligonucleotide primers, the sequence of which are complementary to target regions of genes known to encode for specific microbial functions (e.g. contaminant degradation). In brief, the DNA sample is denatured to produce single stranded DNA, called template DNA, to which the oligonucleotide primers can bind. The enzyme DNA polymerase then adds nucleotide bases to the end of each primer, using the template DNA as a guide to extend the primer thereby producing new double stranded DNA. This process is repeated for a number of cycles to enrich the DNA sample for the desired genes targeted by the oligonucleotide primers. Since each cycle of PCR involves creating two new double stranded DNAs from each DNA molecule present, the amount of DNA theoretically doubles with every cycle of PCR. Therefore, after two cycles the concentration of DNA increases by 2^2-fold, after 3 cycles a 2^3-fold increase, etc. After N cycles, PCR generates a 2^N-fold increase in the target DNA.





From: http://allserv.rug.ac.be/~avierstr/principles/pcr.html

Materials

Template DNA (genomic, plasmid, bacterial colony, etc.)
Primers (resuspended in sterile water or TE to a concentration of **100 mM**)
Buffer (usually **10X**, usually sold with Taq polymerase or you can make your own)
$MgCl_2$ (available in 25mM or 50 mM stocks)
Bovine serum albumin (BSA, **30 mg ml^{-1}** stock)
Taq DNA polymerase
dNTPs (**2.5 mM** working solution)
Note: a 2.5 mM working solution of dNTPs means that the final concentration of each dNTP (dATP, dCTP, dGTP, and dTTP) is 2.5 mM, not that all dNTPs together make 2.5 mM. dNTPs come as 100 mM stocks. Therefore, to make the working solution thaw and add 20 µL of each dNTP to 720 µL of nuclease-free water, mix thoroughly and aliquot in 100 µl volumes. Store at -20°C.
Sterile, nuclease-free water
Gloves
PCR thermalcycler
Pipettes (1-10 µl, 5-50 µl, 20-200 µl, and 100-1000 µl) and aerosol barrier pipette tips
PCR tubes (0.2 ml or 0.5 ml)
Master mix tubes (1.5 ml microcentrifuge tubes)

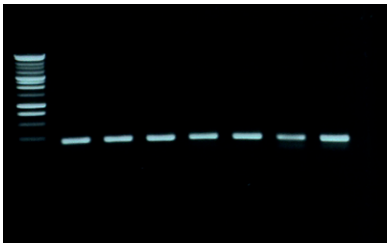
PCR allows the production of more than 10 million copies of a target DNA sequence from only a few molecules. Therefore, PCR is very sensitive to contamination from non-target DNA. Several steps should be taken to reduce the chance for contamination, including:

- Fresh gloves should be worn for DNA purification and each reaction set-up.
- Using aerosol tips (tips with a wad of cotton at the top)
- Limit the amount of close contact with sample tubes and reagents, i.e. don't spit on your workspace.
- DNA sample preparation, reaction mixture assembly and the PCR process, in addition to the subsequent reaction product analysis, should be performed in separate areas.
- The reagents for PCR should be prepared separately and used solely for this purpose.
- Only nuclease-free water should be used in the preparation and suspension of PCR reagents.
- Unless the solution is purchased sterile, autoclaving of all solutions, except dNTPs, primers and *Taq* DNA polymerase is recommended.
- Aliquoted solutions in small portions and store them in designated PCR areas. Aliquots of PCR reagents should be stored separately from DNA samples.

- A control reaction, omitting template DNA, should always be performed, to confirm the absence of contamination.

The protocol in brief

You will perform a PCR reaction on you DNA sample to generate multiple copies of a portion of the 16S rRNA gene. The first step involves setting up a master mix containing enough of the reagents to perform PCR on all of your samples. The master mix is then aliquoted into separate PCR tubes, DNA is added and the tubes are placed into a thermalcycler to perform the DNA replication. Following the reaction, the PCR products will be visualized on an agarose gel (figure below).



PCR products (180 bp) on an agarose gel. A DNA ladder is shown in the first lane

A. Components of the Reaction Mixture

Template DNA (target gene)

This is the DNA/gene that you wish to amplify. The default concentration of DNA used in our laboratory is 1 ng μl^{-1} of PCR reaction. However, this concentration can vary by a few orders of magnitude depending on the target gene concentration and source of DNA.

Higher amounts of template DNA can increase the yield of nonspecific PCR products, but if the fidelity of the reaction is crucial, one should limit both template DNA quantities as well as the number of PCR cycles.

Although several reagents used in DNA extraction and purification protocols such as phenol, EDTA, and proteinase K, can inhibit *Taq* DNA polymerase. Isopropanol precipitation of DNA and treatment of DNA pellets with 70% ethanol is usually effective in removing traces of contaminants from the DNA sample.

PCR Buffer

PCR buffer is necessary to create optimal conditions for activity of *Taq* DNA polymerase. Buffers often contain Tris-Hcl, KCl, and sometimes $MgCl_2$. PCR

buffers are often available in 10X concentration and are sometimes *Taq* formulation-specific.

Although most protocols recommend a final buffer concentration of 1X, increasing the concentration to 1.5X might result in increased PCR product yield.

PCR primers

PCR primers are short fragments of single stranded DNA (15-30 nucleotides in length) that are complementary to DNA sequences that flank the target region of interest. The purpose of PCR primers is to provide a “free” 3’-OH group to which the DNA polymerase can add dNTPs.

The C and G nucleotides should be distributed uniformly throughout of the primer and comprise approximately 40-60% of the bases. More than three G or C nucleotides at the 3'-end of the primer should be avoided, as nonspecific priming may occur.

The primer should not be self-complementary or complementary to any other primer in the reaction mixture, in order to avoid primer-dimer and hairpin formation.

All possible sites of complementarity between primers and the template DNA should be noted.

If primers are degenerate, at least 3 conservative nucleotides must be located at the primer's 3'-end.

The melting temperature of flanking primers should not differ by more than 5°C. Therefore, the GC content and length must be chosen accordingly.

If the primer is shorter than 25 nucleotides, the approx. melting temperature (Tm) is calculated using the following formula:

$$Tm = 4(G + C) + 2(A + T)$$, where

G, **C**, **A**, and **T**, are the number of respective nucleotides in the primer.

If the primer is longer than 25 nucleotides, the melting temperature should be calculated using specialized computer programs where the interactions of adjacent bases, the influence of salt concentration, etc. are evaluated.

The PCR annealing temperature (T_A) should be approximately 5°C lower than the primer melting temperature.

$MgCl_2$
The concentration of $MgCl_2$ influences the stringency of the interaction between the primers and the template DNA. The range of $MgCl_2$ usually tested is from 0.5 - 4 mM in 0.5 mM increments, while the default starting point is often is 1.5 mM.

Low $MgCl_2$ concentrations can help to eliminate non-specific priming and background PCR products and are desirable when fidelity of DNA synthesis is critical. At the same time however, too few Mg^{2+} ions can result in a low yield of PCR product.

High $MgCl_2$ concentrations can help to stabilize interaction of the primers with their intended template if it is not being amplified, but can also result in nonspecific binding and erroneous PCR product formation.

Be aware that some PCR buffers (often sold in 10X stocks) already contain some $MgCl_2$. Therefore the amount of additional $MgCl_2$ must be carefully monitored.

Deoxynucleotide triphosphates (dNTPs)
DNTPs are the nucleotide bases added to the growing DNA strand by the DNA polymerase. The concentration of each dNTP in the reaction mixture is usually 200µM. It is very important to have equal concentrations of each dNTP (dATP, dCTP, dGTP, dTTP), as inaccuracy in the concentration of even a single dNTP dramatically increases the misincorporation level.

***Taq* DNA polymerase**
This DNA polymerase is isolated from the bacterium *Thermus aquaticus*, which lives in hot environments and requires biomolecules that are heat stable. Therefore, *Taq* DNA polymerase can efficiently synthesize DNA under the heat-intensive conditions of the PCR reaction.

Usually 0.02 units of *Taq* DNA polymerase are used per µl of reaction mix. Higher *Taq* DNA Polymerase concentrations may cause synthesis of nonspecific products. However, if inhibitors are present in the reaction mix (e.g., if the template DNA used is not highly purified), higher amounts of *Taq* DNA Polymerase (0.04-0.06 units) may be necessary to obtain a better yield of amplification products.

Bovine serum albumin (BSA, optional)
BSA is a PCR additive that in some cases can enhance the yield of PCR products. It is thought that the BSA sequesters inhibitors that can result in inefficient reactions, however the exact mechanism is unknown. From experience, BSA might or might not result in any yield increase, but usually will not inhibit the reaction itself.

Debate continues whether to use acetylated or non-acetylated BSA. Some researchers attest that non-acetylated BSA inhibits PCR while others report no impact. At this point both have been shown to be effective.

B. The PCR reaction

A typical PCR reaction is performed in a thermalcycler (figure below) and involves an initial DNA denaturation, followed by a number of cycles of denaturation, primer annealing, and product extension. A final DNA extension step completes the reaction.

Initial denaturation

The complete denaturation of the DNA template at the start of the PCR reaction is of key importance to make all potential primer binding sites available. Incomplete denaturation of DNA results in the inefficient utilization of template in the first amplification cycle and in a poor yield of PCR product. The initial denaturation is performed over an interval of 5 minutes at 95°C if the GC content is 50% or less. This interval should be extended up to 10 minutes for GC-rich templates.

Cycles

Denaturation

Denaturation for 45 seconds at 94-95°C is sufficient to completely denature the DNA synthesized in the first amplification cycle. If the amplified DNA has a very high GC content, denaturation time may be increased up to 3-4 minutes.

Primer annealing

The annealing step lasts for approximately 45 seconds, which allows enough time for the primers to locate their complement and anneal to the template DNA strand. The optimal annealing temperature (T_A) is commonly 5°C lower than the

melting temperature of primer-template DNA duplex. In most cases the T_A is between 50 and 65^o C. However, if nonspecific PCR products are obtained in addition to the expected product, the annealing temperature should be optimized by increasing it stepwise by 1-2^o C.

Extension

The processivity of *Taq* DNA polymerase is approximately 150 nucleotides sec^{-1}. Based on this estimate, the extension time necessary to synthesize copies of the 16S rRNA gene (1500 bp) is 10 seconds. However, reaction conditions, template quality, and the presence of inhibitors require that the extension time be increased, usually to between 45-60 seconds for 1500 bp. When larger DNA fragments are amplified, the extending time is usually increased by 1min for each 1000 bp. The extending step is commonly performed at 70-75°C, as the rate of DNA synthesis by *Taq* DNA polymerase is highest at this temperature.

Cycle number

The number of PCR cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product. For less than 10 copies of template DNA, 40 cycles should be performed. If the initial quantity of template DNA is higher, 25-35 cycles are usually sufficient.

Final extension

After the last cycle, the samples are usually incubated at 72°C for 5-15 minutes to fill-in the protruding ends of newly synthesized PCR products. Also, during this step, the terminal transferase activity of *Taq* DNA Polymerase adds extra "A" nucleotides to the 3'-ends of PCR products. Therefore, if PCR fragments are to be cloned into T/A vectors, this step can be prolonged to up to 30 minutes. After the final extension the products may be electrophoresed or stored at –20^o C.

C. Setting up a PCR reaction to amplify a portion of the 16S rRNA gene of bacteria

Below is a portion of the "**BAC2**"spreadsheet that is used in our laboratory to calculate the volume of PCR reagents needed to amplify a portion of the bacterial 16S rRNA gene. Click on the figure to access the actual **BAC2** spreadsheet.

PCR reaction worksheet

date: 12/20/2005

desired DNA conc: 1 ng/ul

reaction size (ul) = 25

rxn # = 7

Mix	Stock conc	des conc	ul per rxn	ul all rxns
buffer (0 mM $MgCl_2$)	10 x	1 x	2.5	17.50
$MgCl_2$	25 mM	2.5 mM	2.5	17.50
BSA	30 mg/ml	1.5 mg/ml	1.3	8.75
each dNTP	2.5 mM	0.2 mM	2.0	14.00
primer 1	100 uM	0.2 uM	0.1	0.35
primer 2	100 uM	0.2 uM	0.1	0.35
H2O			16.1	112.35
taq	5 U ul^{-1}	0.02 U ul^{-1}	0.1	0.70
		total	24.5	171.5
		average DNA volume:		0.50

each rxn:	sample ID	mix	DNA	bulk DNA conc (ng/ul)
1	sample 1	24.50	0.50	50
2	sample 2	24.50	0.50	50
3	sample 3	24.50	0.50	50
4	sample 4	24.50	0.50	50
5	(+) control	24.50	0.50	50
6	(-) control	24.50	#DIV/0!	0

program:	cycle	temp	time
BAC2	1	94	5'
	30	94	45"
		54	45"
		68	45"
	1	72	7'
	1	5	hold

primers:
1 Bac 968 F-GC
2 Bac 1401 R

In general we do not mix the reagents for each individual reaction. Rather, a master mix is made in which enough of each reagent to satisfy all reactions is combined into one tube, and then dispensed among the individual reaction tubes. Template DNA is then added to each tube. This limits pipetting errors as well as the potential for contamination, as the reagent tubes are opened and dispensed from only one time per reaction set-up. Notice also that the number of reactions ("rxn #", in green) calculated by this spreadsheet is one more than the actual number to be run (six vs. seven). This accounts for any pipetting errors and ensures that enough master mix is made to satisfy all of the reactions.

In this example, PCR is being performed on 0.5 μl of DNA (final concentration = 1 ng μl^{-1}) from six samples including one negative control with no DNA added. The PCR volume is 25 μl.

The spreadsheet calculates, based on the stock- and desired- concentrations of each reagent, the volume of reagents needed for each reaction. The spreadsheet then calculates the volume necessary for all the reactions. This is the amount to be added to the master mix. The total number of ml of master mix to be aliquoted into each reaction tube as well as the DNA volume is shown in the right-hand table (24.5 and 0.5 μl, respectively).

This spreadsheet provides information for setting up reactions to be run with primers "Bac 968 F-GC" and "Bac 1401 R" with the BAC2 program of the thermalcycler. Reaction set-up for other primer sets will require a different spreadsheet.

To set up the above PCR reaction, proceed as follows:

1. Wear gloves.

2. Remove the reagents and DNA from the freezer and allow them to thaw on ice. You can let them thaw at room temperature, but be sure to immediately put them back on ice once thawed.

3. Using forceps remove reaction tubes (0.2 ml or 0.5 ml) from the stock bags as well as a master mix tube (1.5 ml) and clearly label.

4. Using aerosol-barrier pipette tips, add nuclease-free water to the master mix tube (112 μl) followed by:

   10X PCR buffer (17.5 μl)
   $MgCl_2$ (17.5 μl)
   BSA (8.75 μl)
   dNTPs (14 μl)
   Primers (0.35 μl each)
      BAC 968 F (5' - AAC GCG AAG AAC CTT AC - 3')
      BAC 1401 R (5' -CGG TGT GTA CAA GAC CC - 3')
   Taq DNA polymerase (0.7 μl).

5. Be sure to change the pipette tip between each reagent and not to accidentally touch the tip to any surface that might contaminate it, as the contaminating DNA could be amplified in the reaction.

6. Briefly vortex the master mix and aliquot 24.5 μl into each of the six reaction tubes.

7. Add 0.5 μl of each DNA sample to the respective PCR tube. Remember, the negative control receives no DNA.

8. Cap the tubes, mix the contents by flicking with your finger, and then briefly (5 seconds) centrifuge the tubes to concentrate the reaction mix at the bottom of the tube.

9. Place the tubes in the PCR thermalcycler, close the lid, and start the **BAC2** program.