- The GC content should be 40-60%. The C and G nucleotides should be distributed uniformly throughout of the primer. 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.
- The melting temperature of flanking primers should not differ by more than 5°C, so the GC content and length must be chosen accordingly.
- 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.
- Estimation of the melting and annealing temperatures of primer:
  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) :G, C, A, T= number of respective nucleotides in the primer.
  Annealing temperature should be approx. 5°C lower than the melting temperature.
  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.

dNTP (deoxynucleotide triphosphates = A, T, G, C)

The concentration of each dNTP in the reaction mixture is usually 200 µM. 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.**

Usually 1-1.5 u of *Taq* DNA Polymerase are used in 50 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 (2-3 u) may be nec

Reaction Mixture Set Up

1. Gently vortex and briefly centrifuge all solutions after thawing.

2. Put PCR tubes on ice. Typically you should have a positive control and a negative control in addition to other templates to amplify. A negative control has no DNA template, and the positive control using a template and primers known to work in PCR.

Reagent	**Final concentration**	**Quantity, for 50 µl of reaction mixture**
Sterile deionized water	-	Variable to make 50 µl total volume
10X *Taq* buffer	1X	5 µl
2 mM dNTP mix	0.2 mM of each	5 µl
Primer I*	0.1-1 µM	1 µl
Primer II*	0.1-1 µM	1 µl
Taq DNA Polymerase	1.25 u / 50 µl	1 µl

Template DNA

The template DNA will be

thermophilic bacteria is used in PCR because it continues to work at the high temperatures

Extending Step.

Usually the extending step is performed at 70-75°C. The rate of DNA synthesis by *Taq* DNA Polymerase is highest at this temperature. Recommended extending time is 1 min for the synthesis of PCR fragments up to 2 kb. When larger DNA fragments are amplified, the extending time is usually increased by 1 min for each 1000 bp.

Number of Cycles.

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 Extending Step.

After the last cycle, the samples are usually incubated at 72°C for 5-15 min 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