# Protocol for PCR with Hot Start *Taq* DNA Polymerase

## How to Avoid Contamination

During PCR, usually more than 10 million copies of a template DNA can be generated. Therefore, care must be taken to avoid contamination with other templates and amplicons that may be present in the laboratory environment. General recommendations to lower the risk of contamination are as follows:

- Prepare your DNA sample, set up the PCR mixture, perform thermal cycling and analyze PCR products in separate areas.
- Set up the PCR mixture in a laminar flow cabinet equipped with UV lamp.
- Wear fresh gloves for DNA purification and reaction set up.
- Use containers dedicated for PCR. Use positive displacement pipettes or pipette tips with aerosol filters to prepare DNA samples and set up the PCR reaction.
- Use PCR-certified reagents, including high quality water (e.g., Water, nuclease-free, #R0581).
- Always perform control reactions without template DNA to check for the absence of contamination.

For detailed instructions for the set-up of a PCR laboratory and its maintenance, refer to PCR Methods and Applications, 3, 2, S1-S14, 1993.

## Reaction Set Up

To prepare several parallel reactions and to minimize the possibility of pipetting errors, prepare a PCR master mix in a single tube by adding water, buffer, dNTPs, primers and Hot Start *Taq* DNA Polymerase. Aliquot the master mix into individual PCR tubes and add template DNA.

1. Gently vortex and briefly centrifuge all solutions after thawing, keep all components on ice.
2. Add in a thin-wall tube at room temperature:

| Component | Volume per 50µl* of reaction | Final concentration |
|---|---|---|
| **Water, nuclease-free** (#R0581) | variable | – |
| **10X Hot Start PCR Buffer** | 5µl | 1X |
| **dNTP Mix, 2mM each** (#R0241) | 5µl | 0.2mM each |
| **Primer I** | variable | 0.1-1µM |
| **Primer II** | variable | 0.1-1µM |
| **25mM $MgCl_2$** | variable** | 1.5-4mM |
| **Hot Start *Taq* DNA Polymerase** | 0.25-0.4µl*** | 1.25 -2.0u/50µl |
| **Template DNA** | variable | 10pg-1µg |

* Reaction volume can be scaled up or down as long as the final concentrations of the reaction components remain the same.

** $Mg^{2+}$ concentration must be optimized for maximal PCR yields. The following table can be used to optimize the concentration of $MgCl_2$ in the PCR mixture:

| Final concentration | 1.5mM | 1.75mM | 2.0mM | 2.5mM | 3.0mM | 4.0mM |
|---|---|---|---|---|---|---|
| **Volume of 25mM $MgCl_2$** | 3µl | 3.5µl | 4µl | 5µl | 6µl | 8µl |

*** 1.25 units are sufficient for amplifying most targets. Increasing Hot Start Taq DNA Polymerase concentration may result in higher yields of specific product.

3. Gently vortex and spin down to collect drops.
4. When using a thermal cycler without a heated lid, overlay the reaction mixture with 25µl of mineral oil.
5. Begin thermal cycling.

**Recommended thermal cycling conditions:**

| Step | Temperature, °C | Time, min | Number of cycles |
|---|---|---|---|
| **Initial Denaturation/ Enzyme activation** | 95 | 2-4 | 1 |
| **Denaturation** | 95 | 0.5-1 | 25-40 |
| **Annealing** | 37-68 | 0.5-1 | |
| **Extension** | 72 | 1min/kb | |
| **Final Extension** | 72 | 5-15 | 1 |

## Components of the Reaction Mixture

### Template DNA

Optimal amounts of template DNA in the 50µl reaction volume are in the 0.01-1ng range for both plasmid and phage DNA, and in the 0.1-1µg range for genomic DNA. Higher amounts of template increase the risk of generation of non-specific PCR products. Lower amounts of template reduce the accuracy of the amplification.

All routine DNA purification methods are suitable for template preparation (e.g., Genomic DNA Purification Kit (#K0512), GeneJET™ Plasmid Miniprep Kit (#K0501)). Trace amounts of certain agents used for DNA purification, such as phenol, EDTA and proteinase K, can inhibit thermostable DNA polymerase. Ethanol precipitation and repeated washes of the DNA pellet with 70% ethanol is usually sufficient to remove trace contaminants from DNA samples.

### Primers

**Guidelines for primer design:**

- PCR primers are generally 15-30 nucleotides long.
- Optimal GC content of the primer is 40-60%. Ideally, C and G nucleotides should be distributed uniformly along the primer.
- Avoid placing more than three G or C nucleotides at the 3'-end to lower the risk of non-specific priming.
- Avoid primer self-complementarity or complementarity between the primers to prevent hairpin formation and primer dimerization.
- Check for possible sites of non-desirable complementarity between primers and the template DNA.
- When designing degenerate primers, place at least 3 conservative nucleotides at the 3'-end.
- Differences in melting temperatures ($T_m$) of the two primers should not exceed 5°C.

**Estimation of primer melting temperature:**

- If the primer contains less than 25 nucleotides, the approx. melting temperature ($T_m$) can be calculated using the following equation:

$$T_m = 4\,(G + C) + 2\,(A + T),$$

where G, C, A, T represent number of respective nucleotides in the primer.

- If the primer contains more than 25 nucleotides specialized computer programs are recommended to account for interactions between adjacent bases, the effect of salt concentration, etc.
- When introducing restriction enzyme sites into primers for subsequent digestion and cloning of a PCR product, refer to the Table "Cleavage efficiency close to the termini of PCR fragments" to define the number of extra bases required for efficient cleavage.

**Primer concentration:**

- The recommended concentration range is 0.1-1.0µM. Lower primer concentrations may result in lower PCR yield, while higher primer concentrations increase the risk of non-specific amplification.

### $MgCl_2$ Concentration

Due to the binding of $Mg^{2+}$ to dNTPs, primers and DNA templates, $MgCl_2$ concentration needs to be optimized for maximal PCR yield. The recommended concentration range is 1.5-4mM. If the $Mg^{2+}$ concentration is too low, the yield of PCR product could be reduced. Non-specific PCR products may appear and the PCR fidelity may be reduced if the $Mg^{2+}$ concentration is too high.

For standard PCR with 0.2mM dNTP concentration the recommended starting $MgCl_2$ concentration is 2.0mM.

If the DNA samples contain EDTA or other metal chelators, the $Mg^{2+}$ ion concentration in the PCR mixture should be increased accordingly (one molecule of EDTA binds one $Mg^{2+}$ (1)).

### dNTPs

The recommended concentration of each dNTP is 0.2mM. It is very important to have equal concentrations of all nucleotides (dATP, dCTP, dGTP and dTTP). If the nucleotide concentrations are not balanced, the PCR error rate may increase dramatically.

To achieve 0.2mM concentration of each dNTP in the PCR mixture, use the following volumes of dNTP Mixes from Fermentas:

| Volume of PCR mixture | dNTP Mix, 2mM each #R0241 | dNTP Mix, 10mM each #R0191 | dNTP Mix, 25mM each #R1121 |
|---|---|---|---|
| 50µl | 5µl | 1µl | 0.4µl |
| 25µl | 2.5µl | 0.5µl | 0.2µl |

### Hot Start *Taq* DNA Polymerase

1.25-2.0u of Hot Start *Taq* DNA Polymerase is generally recommended for a 50µl of PCR mixture volume. It may be necessary to increase the amount of Hot Start *Taq* DNA Polymerase to 3-5u if the PCR mixture contains inhibitors due to contamination of the template DNA.

## Cycling Conditions

Amplification parameters greatly depend on the template, primers and parameters of a thermal cycler. At Fermentas, all functional PCR tests are performed on the GeneAmp® PCR System 9700.

### Initial DNA Denaturation/ Enzyme Activation

This step ensures complete denaturation of template DNA and activation of Hot Start *Taq* DNA polymerase at the beginning of PCR. 4min at 95°C are optimal for this step, however shorter times down to 2min also give good results. For GC-rich DNA templates, this step may be prolonged to 10min.

### DNA Denaturation

In most conditions, a 0.5-1min DNA denaturation at 95°C is sufficient. For GC-rich DNA templates, this step may be increased to 3-4min. DNA denaturation can also be enhanced by the addition of either 10-15% glycerol or 10% DMSO, 5% formamide or 1.7-2M Betaine. The melting temperature of the primer-template complex decreases significantly in the presence of these reagents. Therefore, the annealing temperature must be adjusted accordingly. Additionally, 10% DMSO and 5% formamide inhibit DNA polymerase activity by 50%. Thus, the amount of enzyme should be increased, if these additives are used.

### Primer Annealing

In most conditions, the annealing temperature should be 5°C lower than the primer-template melting temperature ($T_m$). Annealing for 0.5-1min is usually sufficient. If non-specific PCR products appear, the annealing temperature should be optimized stepwise in 1-2°C increments. The annealing temperature must also be adjusted when additives (glycerol, DMSO, formamide or Betaine), which change the melting temperature of the primer-template complex are used.

### Extension

The extension step is performed at 72°C. As a general rule, the extension time with Hot Start *Taq* DNA Polymerase is 1min per 1kb of the DNA fragment.

### Number of Cycles

The number of cycles may vary depending on the amount of template DNA in the PCR mixture and the expected yield of PCR product. If less than 10 copies of the template are present in the reaction, approximately 40 cycles are required. With higher template amounts 25-35 cycles are sufficient.

### Final Extension

After the last cycle, it is recommended to incubate the PCR mixture at 72°C for 5-15min to fill-in the protruding ends of reaction products. If the PCR product is to be cloned into TA vectors (for instance, using InsTAclone™ PCR Cloning Kit (#K1213)), the final extension step can be extended to 30min. Hot Start *Taq* DNA Polymerase adds an extra "dA" to the 3'-ends of PCR products during this step.

### Reference

1. David Harvey, Modern Analytical Chemistry, Mc Graw Hill, p.315, 2000.