Protocol

Long PCR
Joseph Sambrook and David W. Russell

This protocol was adapted from Molecular Cloning, 3rd edition, by Joseph Sambrook and David W. Russell. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2001

INTRODUCTION

The following protocol can be used to amplify DNA up to 25 kb in length. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

MATERIALS

- 10x Long PCR buffer
- Chloroform
- Forward primers (20 µM) in H2O
- Reverse primers (20 µM) in H2O (20 pmoles/µl)
- Template DNA

Long PCR works well on a variety of templates including recombinant PACs, BACs, cosmids, and bacteriophage λ clones, as well as high-molecular-weight genomic DNAs. However, the quality of the DNA is paramount. The average length of the template DNAs (assayed by agarose gel or pulsed-field gel electrophoresis) should be at least three times greater than the length of the desired PCR product. The DNAs should also be extensively purified by equilibrium density centrifugation in CsCl gradients, followed by dialysis against TE (pH 8.0) (please see Purification of Closed Circular DNA by Equilibrium Centrifugation in CsCl-Ethidium Bromide Gradients: Continuous Gradients).

Thermostable DNA polymerase mix

Klentaq1 can be obtained from AB Peptides (St. Louis, Missouri), and Pfu polymerase can be obtained from Stratagene. A typical mixture contains 0.187 unit of Pfu and 33.7 units of Klentaq1 in a total volume of 1.2 µl.

dNTP solution (20 mM) containing all four dNTPs (pH 8.0)

METHOD

1. In a thin-walled amplification tube, add and mix in the following order:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x long PCR buffer</td>
<td>5 µl</td>
</tr>
<tr>
<td>20 mM solution of four dNTPs</td>
<td>5 µl</td>
</tr>
<tr>
<td>20 mM forward primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>20 mM reverse primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>thermostable DNA polymerase mix</td>
<td>0.2 µl</td>
</tr>
</tbody>
</table>
Templates purified from individual recombinant clones constructed in bacteriophage λ, cosmid, bacteriophage P1, PAC, and BAC vectors should be used in amounts ranging from 100 pg to 300 ng. Larger amounts of total genomic DNAs are required, usually between 100 ng and 1 µg per reaction. The optimum amount of template and the optimum ratio of primers:template should be ascertained empirically for each new preparation of DNA.

Primers used for long PCR are generally slightly longer (25-30 nucleotides) than those used for standard PCR. It is particularly important to strive for equality between the melting temperatures of the two primers. If the difference in melting temperatures exceeds one centigrade degree, mispriming and preferential amplification of one strand may become a problem.

2. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Alternatively, place a bead of paraffin wax into the tube if using a hot start PCR. Place the tubes or the microtiter plate in the thermal cycler. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

<table>
<thead>
<tr>
<th>Cycle Number</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Polymerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 cycles</td>
<td>1 min at 94°C</td>
<td>1 min at 60-67°C</td>
<td>5-20 min at 68°C</td>
</tr>
</tbody>
</table>

Times and temperatures may need to be adapted to suit the particular reaction conditions.

The temperature used for the annealing step depends on the melting temperature of the oligonucleotide primers. Because the primers used in long PCR are generally 27-30 nucleotides in length, the annealing temperatures used in long PCR can be considerably higher than those used in standard PCR.

3. If mineral oil was used to overlay the reaction (Step 2), remove the oil from the sample by extraction with 150 µl of chloroform.

The aqueous phase, which contains the amplified DNA, will form a micelle near the meniscus. The micelle can be transferred to a fresh tube with an automatic micropipette.

IMPORTANT
Do not attempt chloroform extractions in microtiter plates. The plastic used in these plates is not resistant to organic solvents.

4. Analyze an aliquot of the aqueous phase by electrophoresis through an agarose gel using markers of an appropriate size (Agarose Gel Electrophoresis). In many cases, the amount of amplified product may be too small to be detected by conventional staining with ethidium bromide. In this case, stain the DNA in the gel with SYBR Gold (Detection of DNA in Agarose Gels) or transfer to a nylon or nitrocellulose filter and probe by Southern hybridization (please see Southern Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Membranes).

REFERENCES

Chloroform (CHCl₃)

Chloroform (CHCl₃) is irritating to the skin, eyes, mucous membranes, and respiratory tract. It is a carcinogen and may damage the liver and kidneys. It is also volatile. Avoid breathing the vapors. Wear appropriate gloves and safety glasses. Always use in a chemical fume hood.

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**Recipe**

**Long PCR buffer**

- 500 mM Tris-Cl (pH 9.0 at room temperature)
- 160 mM ammonium sulfate
- 1.5 mg/ml bovine serum albumin
- 25 mM MgCl₂

Sterilize the 10x buffer by filtration through an 0.22-µm membrane. Divide the sterile buffer into aliquots and store the aliquots at -20°C.

KCl (100 mM) can be used in place of ammonium sulfate in the 10x long PCR buffer, and gelatin can be used at a concentration of 0.01% in the final reaction in place of bovine serum albumin.

Additional components to improve the efficiency of long PCR include glycerol at a concentration of 5% (v/v) in the final reaction mixture to promote separation of DNA strands at lower temperatures, and EDTA at a concentration of 0.75 mM in the final reaction mixture to chelate divalent cations such as Mn²⁺ that might promote scission of DNA strands.

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**Recipe**

**dNTP solution**

Dissolve each dNTP (deoxyribonucleoside triphosphates) in H₂O at an approximate concentration of 100 mM. Use 0.05 M Tris base and a micropipette to adjust the pH of each of the solutions to 7.0 (use pH paper to check the pH). Dilute an aliquot of the neutralized dNTP appropriately, and read the optical density at the wavelengths given in the table below. Calculate the actual concentration of each dNTP. Dilute the solutions with H₂O to a final concentration of 50 mM dNTP. Store each separately at -70°C in small aliquots. For polymerase chain reactions (PCRs), adjust the dNTP solution to pH 8.0 with 2 N NaOH. Commercially available solutions of PCR-grade dNTPs require no adjustment.

<table>
<thead>
<tr>
<th>Base</th>
<th>wavelength (nm)</th>
<th>Extinction Coefficient (E) (M⁻¹cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>259</td>
<td>1.54 x 10⁴</td>
</tr>
<tr>
<td>G</td>
<td>253</td>
<td>1.37 x 10⁴</td>
</tr>
<tr>
<td>C</td>
<td>271</td>
<td>9.10 x 10³</td>
</tr>
<tr>
<td>T</td>
<td>267</td>
<td>9.60 x 10³</td>
</tr>
</tbody>
</table>

For a cuvette with a path length of 1 cm, absorbance = EM. 100 mM stock solutions of each dNTP are commercially available (Pharmacia).