Protocol

Blunt-end Cloning of PCR Products

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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

Incubation of a blunt-end ligation reaction in the presence of an excess amount of an appropriate restriction enzyme can dramatically increase the yield of recombinant plasmids. The role of the restriction enzyme is to cleave circular and linear concatemers at restriction sites that are re-formed when linear, blunt-ended plasmid molecules ligate to themselves. In almost all cases, ligation of the PCR product to the plasmid destroys the restriction site. The constant reclamation of vector molecules drives the equilibrium of the ligation reaction strongly in favor of the recombinants between vector and blunt-ended PCR product.

MATERIALS

- 10x Universal KGB buffer
- ATP (10 mM)

  Omit ATP from the ligation reaction in Step 2 if the ligation buffer contains ATP.

- Bacteriophage T4 DNA ligase
- Bacteriophage T4 DNA polymerase

  Do not use the Klenow fragment of E. coli DNA polymerase.

- Closed circular plasmid DNA (50 µg/ml)

  Choose a plasmid vector containing a single site for a restriction enzyme that generates blunt ends (e.g., Smal, SrfI, and EcoRV). The plasmid vector and its bacterial host should carry a blue/white screening system.

- Restriction endonuclease for cloning

  The restriction enzyme should generate blunt ends, cleave the vector once, and not cleave the amplified DNA (please see Step 1).

- Restriction endonucleases

  Please see Step 4.

- Target DNA (25 µg/ml), amplified by PCR.

  When the PCR mixture contains more than one or two bands of amplified DNA, purify the target fragment by electrophoresis through low melting/gelling temperature agarose (please see Recovery of DNA from Low-melting-temperature Agarose Gels: Organic Extraction). If not purified by gel electrophoresis, PCR-amplified DNA should be prepared for ligation by extraction with phenol:chloroform and ultrafiltration through a Centricon-100 filter (please see Removal of Oligonucleotides and Excess dNTPs from Amplified DNA by Ultrafiltration).

- dNTP solution (2 mM) containing all four dNTPs
METHOD

1. In a microcentrifuge tube, mix the following in the order shown:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
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<tbody>
<tr>
<td>50 µg/ml closed circular plasmid vector</td>
<td>1 µl</td>
</tr>
<tr>
<td>25 µg/ml amplified target DNA</td>
<td>8 µl</td>
</tr>
<tr>
<td>10x universal KGB buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>H₂O (please see note below)</td>
<td>5 µl</td>
</tr>
<tr>
<td>10 mM ATP</td>
<td>1 µl</td>
</tr>
<tr>
<td>2 mM dNTPs</td>
<td>1 µl</td>
</tr>
<tr>
<td>restriction enzyme</td>
<td>2 units</td>
</tr>
<tr>
<td>T4 DNA polymerase</td>
<td>1 unit</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>3 units</td>
</tr>
</tbody>
</table>

Adjust the amount of H₂O added so that the final reaction volume is 20 µl.
Set up a control reaction that contains all of the reagents listed above except the amplified target DNA.

2. Incubate the ligation mixture for 4 hours at 22°C.

3. Dilute 5 µl of each of the two ligation mixtures with 10 µl of H₂O and transform a suitable strain of competent *E. coli* to antibiotic resistance as described in Preparation and Transformation of Competent *E. coli* Using Calcium Chloride or Transformation of *E. coli* by Electroporation. Plate the transformed cultures on media containing IPTG and X-gal (please see Screening Bacterial Colonies Using X-gal and IPTG: α-Complementation) and the appropriate antibiotic.

4. Calculate the number of colonies obtained from each of the ligation mixtures. Pick a number of white colonies obtained by transformation with the ligation reaction containing the target DNA. Confirm the presence of the amplified fragment by (i) isolating the plasmid DNAs and digesting them with restriction enzymes whose sites flank the insert in the multiple cloning site or (ii) colony PCR (Rapid Characterization of DNAs Cloned in Prokaryotic Vectors).

5. Fractionate the restricted DNA by electrophoresis through an agarose gel using appropriate DNA size markers. Measure the size of the cloned fragments.

6. Confirm the identity of the cloned fragments by DNA sequencing (Cycle Sequencing: Dideoxy-mediated Sequencing Reactions Using PCR and End-labeled Primers), restriction mapping, or Southern hybridization (Southern Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Membranes).

REFERENCES


Recipe

ATP
Dissolve 0.55 g of solid ATP (disodium salt) in 10 mL of 25 mM Tris-Cl (pH 8.0). Store the 100 mM ATP solution in small aliquots at -20°C.

Recipe

Universal KGB Buffer

1 M potassium acetate
250 mM Tris-acetate (pH 7.6)
100 mM magnesium acetate tetrahydrate
5 mM β-mercaptoethanol
0.1 mg/ml bovine serum albumin

Store the 10x restriction endonuclease buffer in aliquots at -20°C.

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⁻¹ cm⁻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).