Cloning PCR Products by Addition of Restriction Sites to the Termini of Amplified DNA

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INTRODUCTION

Pairs of oligonucleotide primers used in PCR are often designed with restriction sites in their 5’ regions. In many cases, the sites are different in the two primers. In this case, amplification generates a target fragment whose termini now carry new restriction sites that can be used for directional cloning into plasmid vectors. The purified fragment and the vector are digested with the appropriate restriction enzymes, ligated together, and transformed into E. coli.

MATERIALS

Bacteriophage T4 DNA ligase

Chloroform

Optional, please see Step 7.

EDTA (0.5 M, pH 8.0)

Ethanol

Optional, please see Step 5.

Forward primer (20 µM) in H2O

Phenol:chloroform (1:1, v/v)

Plasmid DNA cleaved with the appropriate restriction enzyme(s) and purified by gel electrophoresis

If the linearized plasmid DNA carries compatible termini that can be ligated to each other, use alkaline phosphatase to remove the 5’-phosphate groups and suppress self-ligation (please see Dephosphorylation of Plasmid DNA).

Restriction endonucleases

Please see Step 4.

Reverse primer (20 µM) in H2O

Sodium acetate (3 M, pH 5.2)

TE (pH 7.5)

Target DNA

Analyze the sequence of the target DNA for the presence of suitable restriction sites. Clone the DNA to be digested into a plasmid or bacteriophage vector that contains as few nonessential sequences as possible. Analyze an aliquot of the preparation of recombinant plasmid DNA that is to be used as a substrate for mutagenesis by agarose gel electrophoresis in TAE buffer (please see Agarose Gel Electrophoresis). The plasmid must be >90% superhelical.
molecules. Repurify the preparation if any linear or >10% nicked or relaxed plasmids are detected.

Because exonuclease III will initiate digestion from single-strand nicks, it is important that the template DNA consist predominantly of closed circular molecules. Purification of the template has the added advantage of removing small pieces of DNA and RNA from the closed circular DNA preparation. These can interfere with digestion by exonuclease III.

METHOD

1. Design and synthesize the appropriate oligonucleotide primers. Use these forward and reverse primers to set up and carry out four identical amplification reactions (50-µl volume) to amplify the target fragment (please see The Basic Polymerase Chain Reaction). Combine the four PCRs, which, in aggregate, should contain 200-500 ng of the desired amplification product.

   Design forward and reverse primers carrying the appropriate restriction sites. The 3' end of each primer should be an exact complement of approx. 15 consecutive bases at a selected site in the target DNA. The 5' terminus of each primer serves as a clamp to hold together the termini of the amplified DNA and to provide a landing site for the restriction enzyme. The clamp should be 3-10 nucleotides in length. The mid-portion of the primer contains the recognition site for the restriction enzyme. Each primer should therefore be 24-31 nucleotides in length and contain approximately equal numbers of the four bases, with a balanced distribution of G and C residues and a low propensity to form stable secondary structures.

2. If 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, prepare PCR-amplified DNA 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). Dissolve in TE (pH 7.5) at a concentration of 25 µg/ml.

3. In a reaction volume of 20 µl, digest approx. 100 ng of purified PCR product with 1.0-2.0 units of the relevant restriction enzyme(s). Incubate the reactions for 1 hour at the optimum temperature for digestion.

4. At the end of the digestion, adjust the volume to 100 µl with H₂O, and add 0.5 M EDTA to a final concentration of 5 mM. Extract once with phenol:chloroform and once with chloroform.

5. Transfer the aqueous phase to a fresh tube and add 3 M sodium acetate (pH 5.2) to achieve a final concentration of 0.3 M. Add 2 volumes of ethanol. Store the mixture for 30 minutes at 0°C.

6. Recover the precipitated DNA by centrifugation at maximum speed for 5 minutes at 4°C in a microcentrifuge. Discard the supernatant, and then wash the pellet with 70% ethanol. Centrifuge again, remove the supernatant, and allow the DNA to dry.

7. Dissolve the DNA in 10 µl of H₂O.

8. In a microcentrifuge tube, set up the following ligation mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µg/ml amplified target DNA</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>plasmid DNA</td>
<td>20 ng</td>
</tr>
<tr>
<td>10x ligation buffer</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1 unit</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 10 µl</td>
</tr>
</tbody>
</table>
If necessary, add ATP to a final concentration of 1 mM.

When directional cloning is used, the ligation mixture should contain an approx. 1:1 molar ratio of purified target DNA to cleaved plasmid vector.

Set up a control reaction that contains all the reagents listed above except the amplified target DNA.

9. Incubate the ligation mixtures for 4 hours at 16°C.

10. Dilute 5 µl of each of the two ligation mixtures with 10 µl of H2O and transform a suitable strain of competent \textit{E. coli} to antibiotic resistance as described in Preparation and Transformation of Competent \textit{E. coli} Using Calcium Chloride or Transformation of \textit{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.

11. 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).

\textit{In different experiments, the ratio of blue:white colonies can vary between 1:5 and 2:1.}

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

13. Confirm the identity of the cloned fragments by DNA sequencing, restriction mapping or Southern hybridization.

**REFERENCES**


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**Caution**

**Chloroform (CHCl\textsubscript{3})**

Chloroform (CHCl\textsubscript{3}) 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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**Caution**

**Phenol:chloroform**

Phenol is extremely toxic, highly corrosive, and can cause severe burns. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves, goggles, and protective clothing. Always use in a chemical fume hood. Rinse any areas of skin that come in contact with phenol with a large volume of water and wash with soap and water; do not use ethanol!
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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**Caution**

**Sodium acetate (NaOAc)**

Sodium acetate (NaOAc), see Acetic acid

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**Recipe**

**EDTA**

To prepare EDTA at 0.5 M (pH 8.0): Add 186.1 g of disodium EDTA•2H₂O to 800 mL of H₂O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.

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**Recipe**

**Sodium acetate**

To prepare a 3 M solution: Dissolve 408.3 g of sodium acetate•3H₂O in 800 mL of H₂O. Adjust the pH to 5.2 with glacial acetic acid or to 7.0 with dilute acetic acid. Adjust the volume to 1 L with H₂O. Dispense into aliquots and sterilize by autoclaving.

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**Recipe**

**TE buffer, 10X**

- 100 mM Tris-Cl (desired pH)
- 10 mM EDTA (pH 8.0)

Sterilize solutions by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle. Store the buffer at room temperature.
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