Directional Cloning of PCR Products

Gina L. Costa and Michael P. Weiner


INTRODUCTION

This protocol is for directional blunt-end cloning of DNA fragments. The target DNA is PCR-amplified, 3'-extensions are polished with Pfu DNA polymerase, and the amplicon is ligated to a blunt-ended plasmid DNA. The products of the ligation reaction are used to transform competent Escherichia coli. A restriction enzyme is added to the ligation reaction (Liu and Schwartz 1992) to relinearize any self-reлинаting vector DNA.

Directional cloning is achieved by creating a monophosphorylated vector and a monophosphorylated insert. In the desired orientation, ligation results in a single-nicked, circular molecule. In the undesired, opposite orientation, the ligation results in a linear molecule that transforms E. coli with a drastically reduced efficiency. A monophosphorylated vector is created by enzymatically treating the vector with a restriction endonuclease, removing the exposed 5' phosphates with an alkaline phosphatase, and subsequently digesting the vector with a second restriction endonuclease. A monophosphorylated insert is created using one 5'-phosphorylated primer that can be either machine-synthesized or obtained by kinase treatment.

MATERIALS

Reagents

Alkaline phosphatase (0.1-0.2 units)

Commercially available molecular-biology-grade alkaline phosphatase often contains nuclease contamination. We recommend the use of bacterial alkaline phosphatase that has been purified devoid of contaminating nucleases and specifically quality-controlled for use in the PCR-Script assay.

Ammonium acetate, 4 M (optional, see Step 17)

ATP (10 mM)

Bacteriophage T4 polynucleotide kinase (10 units)

Bacteriophage T4 polynucleotide kinase buffer

Betaine solution, PCR-grade (Sigma)

Blunt-end restriction endonucleases, e.g., SrfI and SmaI (10-20 units)

Chloroform:isoamyl alcohol (24:1, v/v)

Cloning vector

dNTP stock solution (10 mM containing all four dNTPs, each at 2.5 mM)

Ethanol, 100% and 70%, room temperature (optional, see Step 17)
Ethanol, ice cold

**PCR fragment ligation buffer (10X)**

**LiCl (10 M)**

Oligonucleotide primers

**PCR buffer (10X, as supplied by enzyme manufacturer)**

**PCR enhancer; e.g., Taq Extender PCR additive (Stratagene) (optional, see Step 14)**

**Pfu DNA polymerase (Stratagene)**

**Phenol:chloroform:isoamyl alcohol (25:24:1)**

**STE buffer (10X) (optional, see Step 17)**

**T4 DNA ligase (4 units)**

**TE**

Template DNA

**Thermostable DNA polymerase (5 units); e.g., Taq DNA polymerase**

**Universal restriction digestion buffer (10X)**

*A buffer that is compatible with the first restriction endonuclease digestion as well as the alkaline phosphatase dephosphorylation should be used to optimize the enzymatic processing of the vector DNA.*

**Equipment**

Equipment for agarose gel electrophoresis (see *Agarose Gel Electrophoresis*)

Equipment for transformation of competent *E. coli* (see, e.g., *The Hanahan Method for Preparation and Transformation of Competent E. coli: High-Efficiency Transformation*)

*Optional:* Equipment for screening bacterial colonies using X-gal and IPTG (see *Screening Bacterial Colonies Using X-gal and IPTG: α-Complementation*).

**Thermal cycler**

**Vacuum concentrator**

**Water baths, preset to 37°C, 65°C, 72°C, 95°C, and to the appropriate temperature for restriction digestion and alkaline phosphatase treatment**

**METHOD**

**Preparation of Directional Cloning Vectors**

1. Digest the appropriate vector DNA with the first blunt-end restriction endonuclease (e.g., *Srfl*) as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector DNA (1 µg/µl)</td>
<td>1 µg/µl</td>
</tr>
<tr>
<td>Restriction enzyme (10 units/µl)</td>
<td>1-2 µl</td>
</tr>
<tr>
<td>Universal restriction digestion buffer (10X)</td>
<td>5 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 50 µl</td>
</tr>
</tbody>
</table>

**Directional Cloning of PCR Products -- Costa and Weiner 2006 (1): pdb.p... http://cshprotocols.cshlp.org/cgi/content/full/2006/1/pdb.prot4140?print=true**

2 of 9 4/29/2009 3:49 PM
2. Incubate at the appropriate temperature for 1 hour. Optional: A 1-µl aliquot of the reaction can be run on an agarose gel (see Agarose Gel Electrophoresis) to check for linearization of the vector DNA.

3. Inactivate the restriction enzyme by incubating the reaction for 20 minutes at 65°C. Remove to ice.

4. Add the alkaline phosphatase enzyme (0.1-0.2 units) directly to the heat-treated reaction mixture and incubate according to the manufacturer’s guidelines.

5. Extract the digestion by adding one volume of phenol:chloroform:isoamylalcohol, vortex, centrifuge briefly and transfer the aqueous top phase to a new tube. Add an equal volume of chloroform to the tube and vortex. Centrifuge briefly, and carefully transfer the aqueous phase to a new tube. Heat-treat the extracted DNA for 20 minutes at 65°C to remove any remaining chloroform.

6. Set up a second restriction enzyme digestion containing the processed alkaline phosphatase-treated vector DNA with a downstream blunt-end restriction endonuclease (e.g., SmaI) as follows:

<table>
<thead>
<tr>
<th>Vector DNA (as obtained in Step 5)</th>
<th>15-µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Universal restriction enzyme buffer</td>
<td>3 µl</td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>10-20 units</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 30 µl</td>
</tr>
</tbody>
</table>

Incubate this reaction at the appropriate temperature for 1 hour.

7. Inactivate the restriction enzyme by incubating the reaction for 20 minutes at 65°C. Remove to ice.

8. Precipitate the monophosphorylated DNA with 0.1 volume of 10 M LiCl and 2.5 volumes of ice-cold 100% ethanol. Mix gently and centrifuge at room temperature at 12,000 g for 10 minutes.

9. Following centrifugation, decant the supernatant and dry the DNA pellet in a vacuum concentrator for 10 minutes.

10. Redissolve the DNA in 25 µl of TE buffer. When redissolved into 25 µl of TE, the final concentration of the monophosphorylated DNA should be approximately 10 ng/µl. The monophosphorylated directional cloning vector can be stored at -20°C until use.

**Kinase Treatment of a DNA Primer**

11. Add the following to a microcentrifuge tube:

<table>
<thead>
<tr>
<th>Kinase buffer (10X)</th>
<th>3 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (10 mM)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>T4 DNA kinase (10 units/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Chosen primer</td>
<td>5 µg</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 30 µl</td>
</tr>
</tbody>
</table>

12. Incubate for 1 hour at 37°C.

13. Boil the reaction at 95°C to inactivate the T4 polynucleotide kinase.

**PCR Amplification**

14. Set up a 25-µl reaction in a 0.5-ml sterile, microcentrifuge tube placed on ice. Remember that one of the primers has to be 5’-phosphorylated. Add the following reagents in order:
**DNA polymerase buffer (10X)** 2.5 µl  
**Template DNA (10^5-10^6 target molecules*)** 1-10 µl  
dNTP stock solution (10 mM) 0.5 µl  
**Betaine solution (5 M)** 2.5 µl  
Upstream primer (4 µg/µl) 0.25 µl  
Downstream primer (4 µg/µl) 0.25 µl  
Thermostable DNA polymerase (5 units/µl) 0.25 µl  
**Optional: Taq Extender PCR additive** 5 units  
H₂O to 25 µl

*For 3 x 10^5 targets: 1 µg of human single-copy genomic DNA, 10 ng of yeast DNA, 1% of an M13 plaque.

15. Mix well and carry out the PCR immediately using the following program:

<table>
<thead>
<tr>
<th>Cycle number</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Polymerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4 min at 95°C</td>
<td>2 min at 50°C</td>
<td>2 min at 72°C</td>
</tr>
<tr>
<td>25-30</td>
<td>1 min at 94°C</td>
<td>2 min at 54°C</td>
<td>1 min at 72°C</td>
</tr>
<tr>
<td>Last cycle</td>
<td></td>
<td></td>
<td>10 min at 72°C, then cool to 10°C</td>
</tr>
</tbody>
</table>

16. Check the fidelity and yield of the PCR products by analyzing 1-5 µl of each on an agarose gel alongside suitable control DNAs.

**PCR Product Purification**

17. Optional, purify the PCR products as follows:
   i. Add 0.1 volume of 10X STE buffer.

   ii. Add an equal volume of 4 M ammonium acetate to the sample.

   iii. Add 2.5 volumes of room-temperature-equilibrated 100% ethanol.

   iv. Immediately centrifuge at 12,000g for 10 minutes at room temperature to pellet the DNA. Carefully decant the supernatant.

   v. Add 200 µl of 70% (v/v) ethanol.

   vi. Collect the DNA by centrifuging at 12,000g for 10 minutes at room temperature. Carefully decant the supernatant. Dry the pellet in a vacuum concentrator.

   vii. Resuspend the DNA in the original volume using TE buffer. Store at 4°C until use.

**End-Polishing PCR Products with Pfu DNA Polymerase**

18. Polish the unpurified or purified PCR products according to Steps 18.i or 18.ii below.
   i. For polishing of *unpurified* PCR product, assemble the following reaction in a 0.5-ml microcentrifuge tube:
Unpurified PCR product  | 5-10 µl  
Pfu DNA polymerase (2.5 units) | 1 µl  
H₂O | to 10 µl  

### ii. For polishing of purified PCR product, assemble the following reaction in a 0.5-ml microcentrifuge tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified PCR product</td>
<td>5-10 µl</td>
</tr>
<tr>
<td>Pfu DNA polymerase buffer, 10X</td>
<td>1 µl</td>
</tr>
<tr>
<td>dNTP mix (10 mM total)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Pfu DNA polymerase (2.5 units)</td>
<td>1 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 10 µl</td>
</tr>
</tbody>
</table>

19. Incubate the polishing reaction for 30 minutes at 72°C.

20. Following the 30-minute incubation, remove the reaction to ice.

21. End-polished DNA fragments may be added directly to a ligation reaction.

### Ligation

22. In an autoclaved, sterile 1.5-ml tube, set up the PCR-Script reaction by adding the following reagents in order:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloning vector (10 ng/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Ligation buffer (10X)</td>
<td>1 µl</td>
</tr>
<tr>
<td>ATP (10 mM)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Pfu-polished PCR product insert</td>
<td>1-4 µl</td>
</tr>
<tr>
<td>SrfI restriction endonuclease</td>
<td>5 units</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>4 units</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 10 µl</td>
</tr>
</tbody>
</table>

For ligation, the ideal ratio of insert-to-vector DNA is variable. For sample DNA, a range from 5:1 (when using polished inserts) to 100:1 (when using unpolished inserts) may be necessary. It may be advantageous to optimize conditions for a particular insert using the following equation: pmol ends/µg of DNA = 2 x 10⁶/number of bp.

23. Mix gently and incubate for 1-2 hours at room temperature.

24. Heat-treat the sample for 10 minutes at 65°C.

25. Store the sample on ice until ready to transform competent *E. coli*.

### Transformation

26. Transform competent *E. coli* using any standard method, e.g., The Hanahan Method for Preparation and Transformation of Competent *E. coli*: High-Efficiency Transformation. X-gal/IPTG and colony PCR may be used to screen for bacteria carrying recombinant plasmids (see Screening Bacterial Colonies Using X-gal and IPTG:...
ACKNOWLEDGMENTS

The authors thank John Bauer, Steve Wells, Tim Sanchez, Mark Kaderli, and Bruce Jerpseth for their substantial contributions in experimental design.

REFERENCES


---

**Caution**

Ammonium acetate

Ammonium acetate H$_3$CCOONH$_4$ may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses. Use in a chemical fume hood.

---

**Caution**

Chloroform:isoamyl alcohol

Chloroform (CHCl$_3$) 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.

Isoamyl alcohol may be harmful by inhalation, ingestion, or skin absorption and presents a risk of serious damage to the eyes. Wear appropriate gloves and safety goggles. Keep away from heat, sparks, and open flame.

---

**Caution**

Phenol:chloroform:isoamyl alcohol

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 (CHCl$_3$) 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.

Isoamyl alcohol may be harmful by inhalation, ingestion, or skin absorption and presents a risk of serious damage to the eyes. Wear appropriate gloves and safety goggles. Keep away from heat, sparks, and open flame.

---

**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

Ammonium acetate

To prepare a 10 M solution in 1 L, dissolve 770 g of ammonium acetate in 800 mL of H₂O. Adjust volume to 1 L with H₂O. Sterilize by filtration. Alternatively, to prepare a 100-mL solution, dissolve 77 g of ammonium acetate in 70 mL of H₂O at room temperature. Adjust the volume to 100 mL with H₂O. Sterilize the solution by passing it through a 0.22-µm filter. Store the solution in tightly sealed bottles at 4°C or at room temperature. Ammonium acetate decomposes in hot H₂O and solutions containing it should not be autoclaved.

Recipe

Bacteriophage T4 Polynucleotide Kinase Buffer

- 700 mM Tris-Cl (pH 7.6)
- 100 mM MgCl₂
- 50 mM dithiothreitol

Divide the 10x stock into small aliquots and store frozen at -20°C.

Recipe

LiCl

To prepare 10 M lithium chloride: Dissolve 42.4 g of LiCl in a final volume of 90 mL of H₂O. Adjust the volume of the solution to 100 mL with H₂O. Sterilize the solution by passing it through a 0.22-µm filter, or by autoclaving for 15 minutes at 15 psi (1.05 kg/cm ²) on liquid cycle. Store the solution at 4°C.

Recipe

PCR fragment ligation buffer, 10x

- 250 mM Tris-HCl, pH 7.5
- 100 mM MgCl₂
- 100 mM DTT
- 200 µg/ml BSA
STE buffer
1M NaCl
200 mM Tris-HCl
100 mM EDTA

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.

Recipe

Universal restriction digestion buffer (10X)
1 M potassium acetate
250 mM Tris-acetate, pH 7.6
100 mM magnesium acetate
100 µg/ml BSA
5 mM β-mercaptoethanol

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>
</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).