Protocol

Inverse 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

Inverse PCR is used to amplify and clone unknown DNA that flanks one end of a known DNA sequence and for which no primers are available. The technique involves digestion by a restriction enzyme of a preparation of DNA containing the known sequence and its flanking region. The individual restriction fragments (many thousands in the case of total mammalian genomic DNA) are converted into circles by intramolecular ligation, and the circularized DNA is then used as a template in the PCR. The unknown sequence is amplified by two primers that bind specifically to the known sequence and point in opposite directions. The product of the amplification reaction is a linear DNA fragment containing a single site for the restriction enzyme originally used to digest the DNA. 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 Amplification buffer
  *Include 0.01% (w/v) gelatin in the buffer.*
- ATP (10 mM)
  *Omit ATP from the ligation reaction in Step 2 if the ligation buffer contains ATP.*
- Bacteriophage T4 ligase (1 unit/µl)
- Chloroform
- Ethanol
  *Optional, please see Step 5.*
- Oligonucleotide primer 1 (20 µM) in H2O and Oligonucleotide primer 2 (20 µM) in H2O
  *Each primer should be 20-30 nucleotides in length and should 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. Restriction sites can be added to the 5' ends of the primers to facilitate subsequent cloning.*
- Phenol:chloroform (1:1, v/v)
- Restriction endonucleases
  *Please see Step 4.*
- Sodium acetate (3 M)
- TE (pH 8.0)
Template DNA in 10 mM Tris-Cl (pH 7.6) containing <0.1 mM EDTA.

Inverse PCR requires a circular DNA as template. Steps 1-4 of this protocol describe how such templates can be generated from conventional preparations of linear DNAs, which can be a purified fragment of DNA; a preparation of total genomic DNA, fractionated according to size; a bacteriophage λ cDNA library; an aliquot of a cosmid or bacteriophage P1 genomic library; or any other DNA whose sequence complexity is <10^9 bp.

Thermostable DNA polymerase

For advice on which enzyme to use, please see Table 12-19 in Protocol 5 in the print version of the manual. This protocol has been written with AmpliTaq CS in mind, but it will work well for thermostable enzymes with similar properties.

- Tris-Cl (10 mM, pH 7.6)
- dNTP solution (20 mM) containing all four dNTPs (pH 8.0)

**METHOD**

1. Design and synthesize oligonucleotide primers 1 and 2 based on the known sequence of DNA.

2. Digest 2-5 µg of DNA template (sequence complexity <10^9 bp) with an appropriate restriction enzyme (please see note below). Extract the digested DNA with phenol:chloroform, and then with chloroform alone. Precipitate the DNA with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. Recover the precipitated DNA by centrifugation and dissolve it in TE (pH 8.0) at a concentration of 100 µg/ml.

   Alternatively, heat the digested DNA to 65°C for 15-20 minutes to inactivate the restriction enzyme.

3. In sterile 0.5-ml microcentrifuge tubes, amplification tubes, or the wells of a sterile microtiter plate, set up a series of ligation reactions containing cleaved template DNA at concentrations ranging from 0.1 to 1 µg/ml.

| template DNA | 10 ng to 100 ng |
| 10x ligation buffer | 10 µl |
| 1 unit/µl bacteriophage T4 DNA ligase | 4 µl |
| 10 mM ATP | 10 µl |
| H₂O | to 100 µl |

Incubate the reactions for 12-16 hours at 16°C.

Some commercial ligase buffers contain ATP. When using such buffers, the addition of ATP to the ligation reaction is no longer required.

4. Extract the ligated DNA with phenol:chloroform, and then with chloroform alone. Precipitate the DNA with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. Recover the precipitated DNA by centrifugation and dissolve it in 10 mM Tris (pH 7.6) or H₂O at a concentration of 100 µg/ml.

5. In a sterile 0.5-ml thin-walled amplification tube, add and mix in the following order:

| 10x amplification buffer | 5 µl |
| 20 mM solution of four dNTPs (pH 8.0) | 1 µl |
| 20 µM oligonucleotide primer 1 | 2.5 µl |
Set up two control reactions. In one reaction, include all of the above reagents, except the template DNA. In the other reaction, replace the template with a plasmid of known size, containing the DNA insert from which the oligonucleotide primers were derived. Carry each control reaction through all subsequent steps of the protocol.

6. 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 wax into the tube if using 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>30 cycles</td>
<td>30 sec at 94°C</td>
<td>30 sec at 60°C</td>
<td>2.5 min at 72°C</td>
</tr>
<tr>
<td>Last cycle</td>
<td>30 sec at 94°C</td>
<td>30 sec at 60°C</td>
<td>10 min at 72°C</td>
</tr>
</tbody>
</table>

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

The exact annealing temperature should be established empirically for the primer pairs used in a given amplification reaction. An extended polymerization time (up to 10 minutes per cycle) should be tried if the target DNA is long (>4kb). Alternatively, the use of mutant thermostable DNA polymerases that lack and/or that contain a low level of 3'-exonuclease activity may produce longer templates.

7. Withdraw a sample (5-10 µl) from the test reactions and the control reactions and analyze them by electrophoresis through an agarose (Agarose Gel Electrophoresis) or polyacrylamide gel (Neutral Polyacrylamide Gel Electrophoresis). Include DNA markers of an appropriate size. Stain the gel with ethidium bromide or SYBR Gold (Detection of DNA in Agarose Gels or Detection of DNA in Polyacrylamide Gels by Staining).

A successful amplification reaction should yield a readily visible DNA. The identity of the band can be confirmed by DNA sequencing (please see Cycle Sequencing: Dideoxy-mediated Sequencing Reactions Using PCR and End-labeled Primers), and/or restriction mapping and/or Southern hybridization using probes homologous to the known DNA sequence (please see Southern Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Membranes).

REFERENCES


**Caution**

**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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**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**

**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.

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**Recipe**

**Amplification Buffer, 10X**

- 500 mM KCl
- 100 mM Tris-Cl (pH 8.3 at room temperature)
- 15 mM MgCl₂

Autoclave the 10x buffer for 10 minutes at 15 psi (1.05 kg/cm²) on liquid cycle. Divide the sterile buffer into aliquots and store them at -20°C.
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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Recipe

**Tris-Cl**

- **Tris base**
- **HCl**

To prepare a 1 M solution, dissolve 121.1 g of Tris base in 800 mL of H₂O. Adjust the pH to the desired value by adding concentrated HCl.

<table>
<thead>
<tr>
<th>pH</th>
<th>HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>70 mL</td>
</tr>
<tr>
<td>7.6</td>
<td>60 mL</td>
</tr>
<tr>
<td>8.0</td>
<td>42 mL</td>
</tr>
</tbody>
</table>

Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 L with H₂O. Dispense into aliquots and sterilize by autoclaving.

If the 1 M solution has a yellow color, discard it and obtain Tris of better quality. The pH of Tris solutions is temperature-dependent and decreases ~0.03 pH units for each 1°C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9, and 8.6 at 5°C, 25°C, and 37°C, respectively.

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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).

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