Cloning and Analysis of PCR-generated DNA Fragments

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INTRODUCTION

This chapter presents methods for the improved blunt-end cloning of PCR-generated DNA fragments (Costa et al. 1994a). We show that *Pfu* DNA polymerase polishing of *Taq* DNA polymerase-generated PCR fragments increases the yield and efficiency of cloning. Using a triple primer set consisting of two outside, asymmetrically distanced primers and one fragment-specific primer, one can determine both the presence and orientation of cloned inserts. Application of these methods allows one to generate and clone a fragment in 1 day and to analyze putative clones the next, thereby saving a substantial amount of both time and effort.

REAGENTS

Preparation of Cloning Vector

Cloning kits:

- pCR-Script SK(+) Amp cloning system (Stratagene, cat. no. 211190)
- pCR-Script SK(+) Cam cloning system (Stratagene, cat. no. 211192)
- pCR-Script Direct SK(+) cloning system (Stratagene, cat. no. 211194)

Reagents required:

- Cloning vector (10 ng/μl)
- Blunt-end restriction endonuclease (10–20 units)
  - *Srf*I restriction endonuclease (Stratagene; cat. no. 501064)
  - *Sma*I restriction endonuclease
- 10X Universal buffer (1 M KOAc, 250 mM Tris-acetate, pH 7.6, 100 mM MgOAc, 5 mM β-mercaptoethanol, 100 μg/ml BSA)
- Alkaline phosphatase (0.1–0.2 units)
- Phenol (Tris-buffered)
- Chloroform-isoamyl alcohol (24:1)

Lithium chloride (LiCl; 10 M)
TE buffer (5 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)

Several vectors and vector derivatives have been created for PCR cloning. These include the standard pBluescript-type multiple cloning sites (e.g., pBluescript II, pBK, pBC, pCR-Script Amp, and pCR-Script Cam; Stratagene) and the abbreviated multiple cloning sites as contained in the pCR-Script Direct plasmids (Stratagene) (see Fig. 1) (Bauer et al. 1992; Costa and Weiner 1994a,b; Costa et al. 1994b,c). The abbreviated multiple cloning sites allow the end user to incorporate common restriction enzyme sites into the PCR primer sets without the problem of having the same target sequence occurring in the plasmid vector. It is recommended that the chloramphenicol (cam) derivatives be used when subcloning DNA fragments generated from ampicillin (amp)-resistance-encoding plasmids. This ensures that after *Escherichia coli* transformation recombinant colonies are not the result of parental plasmid transformation.

PROTOCOLS

Bidirectional Cloning Vectors

Blunt-end cloning procedures utilize cloning vectors with blunt ends to capture DNA fragments for bidirectional insertion. Therefore, blunt-end cloning vectors do not require nucleotide overhangs for clonal insertion. Subsequently, blunt DNA fragments, such as PCR products, may be cloned directly. Because blunt-end cloning does not require the addition of extra bases to the primer sets, preexisting primers may be used to generate and clone a DNA fragment. By itself, blunt-end cloning is an inefficient method, with recombinant insertion generally accounting for less than 10% of all transformants (see Fig. 2A). Increased efficiency can be achieved by the inclusion of a restriction enzyme in the ligation reaction as in the pCR-Script method (see Fig. 2B) (Liu and Schwartz 1992).

1. Digest the vector DNA with restriction endonuclease in a 20-µl reaction mixture containing ddH_2O, the appropriate reaction buffer, plasmid DNA (1 µg), and restriction enzyme (10–20 units). Allow the digestion to incubate at the recommended temperature for 1 hour. *Optional:* A 1-µl aliquot of the digestion can be run on an agarose gel to check for linearization of the vector DNA.

2. Phenol/chloroform-extract the restriction digestion. Add an equal volume of Tris-buffered phenol, vortex, and transfer the aqueous top phase to a new tube. Add an equal volume of chloroform to the tube and vortex. Transfer the aqueous top phase to a new tube. Heat-treat the extracted DNA for 20 minutes at 65°C to remove any remaining chloroform (the boiling point of chloroform is 55°C).

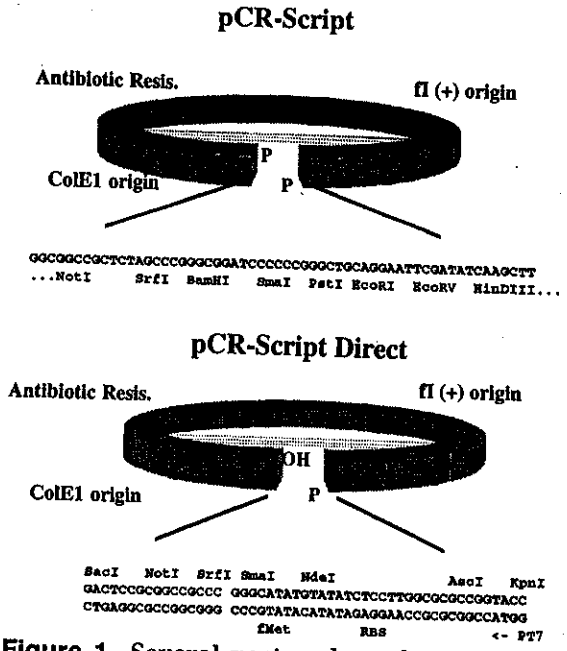


Figure 1 Several vectors have been developed for directional and bidirectional cloning. These include derivatives that encode either chloramphenicol or ampicillin resistance with the modified multiple cloning sites optimized for specific cloning operations (e.g., general subcloning or protein expression).

3. Precipitate the DNA extracted in step 2 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.

4. Following centrifugation, decant the supernatant. Dry the DNA pellet in vacuo for 10 minutes.

5. Resuspend the DNA in 50 µl of TE buffer. When resuspended into 50 µl of TE, the final concentration of the 1 µg of digested DNA should be approximately 10 ng/µl. Store the predigested vector at 4°C.

Directional Cloning Vectors

We relied on previous characterization of T4 DNA ligase and *E. coli* transformation to create a directional cloning method that does not require the addition of extra bases to the primers. First, T4 DNA ligase requires both a 5′ phosphate and a 3′ hydroxyl group to ligate two strands of DNA together efficiently. Second, linear DNA transforms *recBC*-proficient hosts of *E. coli* at a greatly reduced efficiency (it is decreased by approximately four orders of magnitude). It was therefore reasoned that directional cloning could be achieved by creating a

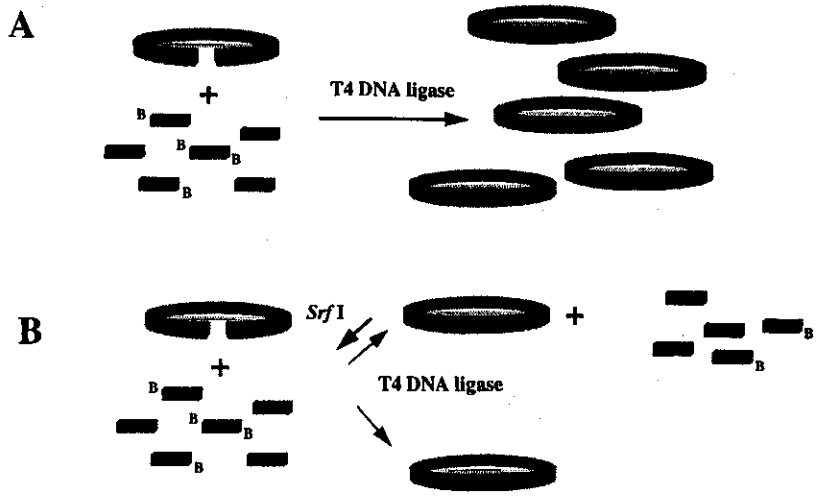


Figure 2 Blunt-end and pCR-Script cloning methods. Methods for standard blunt-end cloning (*A*) include incubation of the PCR product with predigested vector DNA and T4 DNA ligase. More efficient methods (*B*) include the addition of the restriction enzyme (in this example, *Srf* I endonuclease) to regenerate the linearized vector from the self-ligated vector during the ligation reaction.

monophosphorylated vector and a monophosphorylated insert. In the desired orientation, the ligation would result in a single-nicked, circular molecule. In the undesired, opposite orientation, the ligation would result in a linear molecule that would transform *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. Degenerate restriction endonucleases may also be used.

Proper DNA sequence manipulation will enable the enzymatically processed vector to be used in a pCR-Script-type reaction, whereby self-ligated vector is susceptible to restriction by the endonuclease present in the ligation reaction, and the reading frame of the reporter gene is conserved. Owing to the importance of recreating a restriction enzyme site following vector self-ligation, the necessity of using highly purified enzymes for performing the directional and bidirectional cloning protocols as described cannot be overstated. Nuclease contamination must be determined and eliminated prior to performing the described experiments.

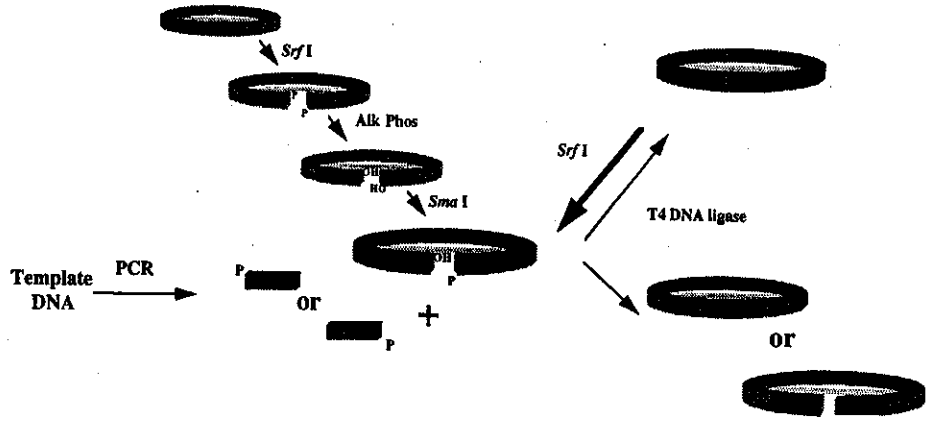
In a specific example, using the pCR-Script Direct directional cloning method, we enzymatically processed an SK(+) multiple cloning site that was engineered to contain both an *Srf* I (5′-GCCC|GGGC-3′) site and an *Sma*I (5′-TCCC|GGGC-3′; where the *Sma*I target sequence is underlined) site (Weiner 1993). The vector was first

digested with *Srf* I, followed by removal of the 5′ phosphates with alkaline phosphatase and a second digestion with *Sma*I. Removal of the short DNA fragment after the *Srf* I-*Sma*I digestions results in the retention of an *Srf* I site (see Fig. 3). Phenotypic selection can still be used, since the reading frame is conserved. The monophosphorylated vector is produced by the general guidelines below.

1. Digest the appropriate vector DNA with the first blunt-end restriction endonuclease in a 50-µl reaction mixture containing ddH_2O, 1X Universal buffer, plasmid DNA (1 µg), and enzyme (10–20 units). Allow the digestion to incubate at the recommended temperature for 1 hour. *Optional:* A 1-µl aliquot of the reaction can be run on an agarose gel to check for linearization of the vector DNA.

   Note: 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.

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



pCR-Script Direct SK(+) Multiple Cloning Site

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P(βGal) ->
SacI    NotI SrfI PstI BamHI  SmaI   NdeI          AscI   KpnI
GACTCCGCGGCCGCCCGGGCTGCAGGATCCCGGGCATATGTATATCTCCTTGGCGCGCCGGTACC
CTGAGGCGCCGGCGGGCCCGACGTCCTAGGGCCCGTATACATATAGAGGAACCGCGCGGCCATGG
                                  fMet       RBS          <- P(T7)
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Figure 3 Monophosphorylation and pCR-Script Direct cloning. The plasmid pCR-Script Direct is digested with the restriction enzyme *Srf* I, treated with alkaline phosphatase to remove the 5′ phosphate groups, and then digested with a second restriction enzyme *Sma*I. Ethanol precipitation was used to remove the small (15-bp) linker. The insert fragment was created using either a machine-synthesized 5′-phosphorylated or kinase-treated primer. The monophosphorylated primer and vector are incubated in the presence of both *Srf* I and T4 DNA ligase. After room temperature incubation, the DNA was used to transform *E. coli.*

3. 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.

   Note: 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.

4. Phenol/chloroform-extract the restriction-digested, alkaline-phosphatase-treated plasmid DNA. Add an equal volume of Tris-buffered phenol, vortex, and transfer the aqueous top phase to a new tube. Add an equal volume of chloroform to the tube and vortex. Transfer the aqueous top phase to a new tube. Heat-treat the extracted DNA for 20 minutes at 65°C to remove any remaining chloroform.

5. Set up a second 30-µl restriction enzyme digestion containing the processed alkaline-phosphatase-treated vector DNA with the downstream blunt-end restriction endonuclease by adding a 15-µl aliquot of phenol/chloroform-extracted DNA, ddH_2O, 1X Universal buffer, and enzyme (10–20 units). Allow this digestion to incubate at the recommended temperature for 1 hour.

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

7. 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.

8. Following centrifugation, decant the supernatant. Dry the DNA pellet in vacuo for 10 minutes.

9. Resuspend the DNA in 25 µl of TE buffer. When resuspended into 25 µl of TE, the final concentration of the monophosphorylated DNA should be approximately 10 ng/µl. The monophosphorylated vector can be stored at 4°C.

REAGENTS

Preparation of Insert

Synthetic oligonucleotide primers

Deoxynucleotide triphosphate mix (dNTP; 10 mM and 100 mM; Pharmacia, cat. no. 27-2094-01)

PCR optimization buffers

Opti-Prime PCR optimization kit (Stratagene, cat. no. 200422)

The PCR Optimizer (Invitrogen, cat. no. K1220-01)

Thermostable DNA polymerases (5–10 units)
Taq DNA polymerase
native *Pfu* DNA polymerase (Stratagene, cat. no. 600135)
cloned *Pfu* DNA polymerase (Stratagene, cat. no. 600153)
Taq Extender PCR additive (Stratagene, cat. no. 600148)
T4 polynucleotide kinase (10 units)
Kinase buffer (10 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol)
ATP (10 mM)
TE buffer
STE buffer (1 M NaCl, 200 mM Tris-HCl, pH 7.5, 100 mM EDTA)
Mineral oil (Sigma, cat. no. M-3516)
Ammonium acetate (NH_4OAc; 4 M)

PCR Primer Design Considerations

Recent studies have shown that many species of DNA polymerases (e.g., T7, modified T7, *Taq*, Vent, *Tth*, and Klenow) exhibit terminal deoxynucleotidyl transferase (TdT) activity (Clark 1988; Hu 1993). The 3′-end nucleotide extension of PCR products by DNA polymerases has been found to be both nucleotide- and polymerase-specific. For example, *Taq* DNA polymerase-generated PCR products would be preferentially modified as follows (+ for extension; – for nonaddition).

3′ Nucleotide extensions associated with *Taq*-generated PCR products (Hu 1993)

3′-End nucleotide	3′-End extension
A	+A (at very low efficiency)
C	+A > +C
G	+G > +A > +C
T	–T > +A

There appears to be no consistent pattern by which bases are added by the polymerase. Therefore, it cannot be assumed that all DNA polymerases can be used to create blunt-end DNA fragments. However, for certain DNA polymerases, the expected 3′-end nucleotide of a PCR product can be controlled by the 5′-end nucleotide of the PCR primer (Hu 1993; Costa and Weiner 1994d).

For directional cloning using a monophosphorylated vector, insert monophosphorylation can be achieved by kinase-treating one primer prior to the PCR. Preferably, this could be achieved by synthesizing a PCR primer with a 5′ phosphate group chemically attached. Synthesis of a PCR primer with a 5′-terminal phosphate group ensures that all single-stranded DNA has been monophosphorylated. An advantage to

kinase treatment is that all preexisting primer sets can be modified for use in directional cloning using a monophosphorylated vector. T4 polynucleotide kinase treatment is a simple and rapid technique.

PROTOCOLS

Primer-kinasing Treatment of a DNA Primer

1. Add the following to a microcentrifuge tube:

   3 µl of 10X kinase buffer
   0.5 µl of 10 mM ATP
   1 µl of T4 DNA kinase (10 units)
   5 µg of primer
   Deionized, distilled H_2O (ddH_2O) to a final volume of 30 µl

2. Incubate for 1 hour at 37°C.

3. Boil the reaction at 95°C to inactivate the T4 DNA kinase.

PCR Parameters

Use of the following standard conditions will amplify most target sequences, although it is recommended that conditions be optimized for each PCR application. Because no specific guidelines exist for choosing which buffer conditions to use for the various types of DNA primer-template systems, it is often advantageous to test a range of PCR buffers. Recently, a number of PCR optimization kits have been created that enable one to test several different buffer compositions (e.g., Opti-Prime PCR Optimization Kit [Stratagene] and The PCR Optimizer [Invitrogen]). By modifying specific buffer components of a PCR, it is possible to improve the yield and specificity of the desired PCR products. In addition, *Taq* Extender PCR additive (Stratagene) improves the PCR amplification of difficult templates and increases the reliability and yield of many PCR targets up to 10 kb in length (Nielson et al. 1994). The *Taq* Extender PCR additive increases the efficiency at which *Taq* DNA polymerase performs extension reactions on specific DNA segments in each cycle of PCR, thus resulting in a greater percentage of the extension reactions reaching completion. The *Taq* Extender should be added to amplification reactions in a unit-equivalent amount equal to that of *Taq* DNA polymerase; the standard *Taq* 10X reaction buffer may be replaced with an optimized *Taq* Extender 10X reaction buffer. Cycling is performed using standard PCR conditions.

PCR Amplification

1. Set up a 100-µl reaction in a 0.5-µl sterile, autoclaved microcentrifuge tube by adding in order:

ddH_2O (for a final volume of 100 ml)	75–84 µl
10X DNA polymerase buffer (for a final 1X volume)	10 µl
Template DNA (10–500 ng of plasmid DNA or 10^5–10^6 target molecules*)	1–10 µl
dNTP mix (250 nM of each dNTP)	2 µl
1 µg of upstream primer (T_m >55°C preferred)	1 µl
1 µg of downstream primer (T_m >55°C preferred)	1 µl
Thermostable DNA polymerase (5 units)	1 µl
suggested:	
Taq DNA polymerase (5 units)	1 µl
Taq Extender PCR additive (5 units)	1 µl

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

2. Mix well and overlay with approximately 75 µl of mineral oil. PCR amplification should be conducted immediately.

3. Perform PCR using the following suggested temperature profile:

For $n = 1$ cycle		
denaturation	4 minutes	94°C
primer annealing	2 minutes	50°C
primer extension	2 minutes	72°C
Follow with $n = 25$–30 cycles		
denaturation	1 minute	94°C
primer annealing	2 minutes	54°C
primer extension	1 minute	72°C
Extend $n = 1$ cycle		
primer extension	10 minutes	72°C

The reactions are stopped by chilling to 4°C.

4. Following thermal cycling, check the PCR products for fidelity and yield by agarose gel analysis. A 10-µl aliquot of PCR product can be monitored by ethidium bromide staining of the DNA fragments following agarose gel electrophoresis. Known amounts of control DNAs should be run as markers for PCR product size and concentration.

Optional PCR Product Purification

The removal of excess PCR primers with selective ammonium acetate precipitation before proceeding with cloning protocols has been

shown to increase the percentage of recombinants. An aliquot of the PCR product can be salted out of solution by the following protocol.

1. Add 0.1 volume of 10x STE buffer.

2. Add an equal volume of 4 M NH_4OAc to the sample.

3. Add 2.5 volumes of room-temperature 100% ethanol.

4. Immediately spin in a centrifuge at 12,000*g* for 10 minutes at room temperature to pellet the DNA. *Carefully* decant the supernatant.

5. Add 200 µl of 70% (v/v) ethanol.

6. Spin in a centrifuge at 12,000*g* for 10 minutes at room temperature. *Carefully* decant the supernatant. Dry the pellet in vacuo.

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

End-polishing PCR Products with *Pfu* DNA Polymerase

Optimizing primer design in accordance with the specific DNA polymerase used can only minimally increase the number of blunt-end fragments produced following PCR. The traditional Klenow polymerase should be *absolutely avoided* for end-polishing because it retains a substantial amount of extendase activity. Fortunately, T4 and *Pfu* DNA polymerases were found not to exhibit any DNA extendase or TdT activity and can be used to create blunt-end fragments following PCR (see Fig. 4) (Hu 1993; Costa and Weiner 1994c,d). PCR polishing is used to remove the 3′-end nucleotide extensions placed on completed PCR products by DNA polymerases. The resulting *Pfu*-polished molecules will ligate into blunt-end cloning vectors at high efficiency in the presence of T4 DNA ligase. End-polishing of PCR products prior to ligation has been shown to increase overall recombinant cloning efficiencies (Costa and Weiner 1994c,d; Weiner et al. 1994).

Pfu DNA polymerase is essentially inactive at temperatures below 50°C. This allows ligation reactions to be done at 4–25°C and to be set up directly from the 72°C *Pfu* polishing step. This eliminates the need to extract the enzyme prior to ligation, as would be required if T4 DNA polymerase were used for polishing. *Pfu* polishing of PCR products generates high-fidelity, blunt-end DNA fragments in 30 minutes using only a small aliquot of the PCR product. *Pfu* polishing is outlined below and can be performed directly following the PCR or following the purification of the desired PCR product.

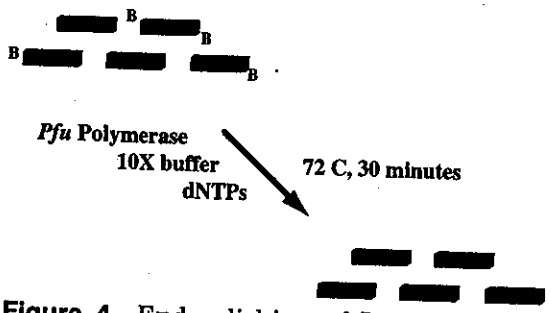

Figure 4 End-polishing of PCR-generated DNA fragments with *Pfu* DNA polymerase is used for increasing the amount of blunt-end DNA available for cloning. See text for protocol.

PROTOCOLS

PCR Polishing: Use of *Pfu* DNA Polymerase

Note: Before PCR polishing, it may be advantageous to verify the PCR products by agarose gel analysis to estimate the approximate concentration and to ensure that the correct PCR products have been created following thermal amplification. PCR polishing is conducted using an aliquot of the PCR amplification reaction and will therefore polish the ends of all DNA fragments present. In a typical 100-µl PCR amplification reaction, 5 µl of product can be used for PCR polishing.

Because routine PCR cloning procedures require the use of a small amount of DNA insert (1–4 µl), end-polishing reactions can be set up directly from the amplification using only 10 µl of PCR product. When end-polishing directly from the PCR, the remaining dNTPs and reaction buffer following thermal cycling are adequate for the polishing reaction (see below). Precipitation of the PCR products may be used for even more efficient end-polishing.

Pfu Polishing of Unpurified PCR Product

1. For polishing PCR-generated DNA fragments, transfer an aliquot of the PCR product directly from the reaction tube into a sterile 0.5-ml microcentrifuge tube and add the following reagents, in order:

   5–10 µl of PCR product
   1 µl of cloned *Pfu* DNA polymerase (2.5 units)
   ddH_2O to a final volume of 10 µl

   Gently mix the components and add a mineral oil overlay.

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

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

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

Pfu Polishing of Purified PCR Product

1. For polishing purified PCR product, transfer an aliquot of the precipitated PCR product into a sterile 0.5-ml microcentrifuge tube and add the following reagents, in order:

   5–10 µl of precipitated PCR product
   1 µl of 10x cloned *Pfu* DNA polymerase buffer
   1 µl of dNTP mix (10 mM total; 2.5 mM each nucleotide triphosphate)
   1 µl of cloned *Pfu* DNA polymerase (2.5 units)
   ddH_2O to a final volume of 10 µl

   Gently mix the components and add a mineral oil overlay.

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

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

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

REAGENTS

Efficient Clonal Ligation and Transformation of Blunt-end PCR Products

Cloning kits:
- pCR-Script SK(+) Amp cloning system (Stratagene, cat. no. 211190)
- pCR-Script SK(+) Cam cloning system (Stratagene, cat. no. 211192)
- pCR-Script Direct SK(+) cloning system (Stratagene, cat. no. 211194)

Reagents required:
- Cloning vector (10 ng/µl)
- Blunt-end insert (100 ng/µl)
- Blunt-end restriction endonuclease (5 units)
  - *Srf*I restriction endonuclease (Stratagene, cat. no. 501064)
- 10x Ligation buffer (250 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 100 mM DTT, 200 µg/ml BSA)
- ATP (10 mM)
- T4 DNA ligase (4 units)
- ddH_2O
- Competent *E. coli* cells
  - XL1-Blue (Stratagene, cat. no. 200236)
  - XL1-Blue MRF′ Kan (Stratagene, cat. no. 200248)
- SOC medium (see Media Preparation, below)
- Luria broth (LB) agar plates (see Media Preparation, below)
- Ampicillin-methicillin LB plates (see Media Preparation, below)
- Chloramphenicol LB plates (see Media Preparation, below)

5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (XGal; 100 mg/ml)
Isopropyl-β-D-thio-galactopyranoside (IPTG; 100 mM)
FALCON 2059 polypropylene tubes

MEDIA PREPARATION

SOC medium (per liter)
20 g tryptone
5 g yeast extract
0.5 g NaCl
water to 900 ml
Autoclave
Mix the following separately:
2.03 g $MgCl_2$
1.2 g $MgSO_4$
3.6 g glucose
Add water to a final volume of 100 ml
Filter-sterilize and add to cooled, autoclaved medium

LB agar (per liter)
10 g NaCl
10 g bacto-tryptone
5 g bacto-yeast extract
20 g bacto-agar
Adjust pH to 7.0 with 5 N NaOH
Add deionized H_2O to a final volume of 1 liter
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

LB-ampicillin-methicillin agar (per liter)
(Use for reduced satellite colony formation)
1 liter of LB agar
Autoclave
Cool to 55°C
Add 20 mg of filter-sterilized ampicillin
Add 80 mg of filter-sterilized methicillin
Pour into petri dishes (~25 ml/100-mm plate)

LB-chloramphenicol agar (per liter)
1 liter of LB agar
Autoclave
Cool to 55°C
Add 30 mg of filter-sterilized chloramphenicol
Pour into petri dishes (~25 ml/100-mm plate)

Ligation Reaction

To increase the efficiency of blunt-end cloning of PCR-generated fragments, it was found that a restriction enzyme added in a functional-unit excess relative to the units of T4 DNA ligase increases the efficiency of the ligation reaction (Liu and Schwartz 1992). This simultaneous restriction digestion and ligation reaction results in an increased efficiency of the blunt-end cloning by two mechanisms. First, as long as the PCR fragment does not, when ligated with the vector, create a restriction enzyme target site, the available circular vector is removed from the overall reaction by recombinant insertion. An increased amount of linear vector is made available during the ligation reaction by the restriction enzyme on self-ligated vector molecules. Second, because linear DNA molecules transform *E. coli* at a greatly reduced efficiency, they do not significantly contribute to the number of colonies observed after transformation. Both of these mechanisms result in a reduced overall transformation efficiency, but because only the linearized, nonrecombinant plasmids are reduced, the overall recombinant efficiency actually increases.

The pCR-Script method uses the restriction enzyme *Srf* I (Simcox et al. 1991). *Srf* I has an octanucleotide recognition sequence (5′-GCCC|GGGC-3′) that is rare and would occur on an average of 1 in 65,000 bp (because of the bias against CpG sequences in some DNA its actual occurrence in mammalian DNA is closer to 1 in 100,000 bp). The target site is blunt-ended and contains an internal 6-base recognition site (5′-CCC|GGG-3′) that can be recognized by another blunt-end restriction enzyme (*Sma*I). This was important to the development of the pCR-Script Direct method because the actual PCR cloning with directionality occurs in a reaction identical to that described for pCR-Script.

The addition of a restriction endonuclease to the ligation reaction allows an overall fourfold increase in clonal efficiency, along with a greatly reduced background. For the bidirectional cloning of PCR-generated DNA fragments, it is recommended to use a pCR-Script-type reaction containing a predigested vector DNA and *Pfu* DNA polymerase-generated or *Pfu*-polished inserts (Costa and Weiner 1994c,d). For the directional cloning of PCR-generated DNA fragments, it is recommended to use a pCR-Script Direct-type cloning reaction with a *Pfu* DNA polymerase-generated or *Pfu*-polished monophosphorylated insert. The procedure is the same for both the pCR-Script bidirectional and the pCR-Script Direct directional cloning.

PROTOCOL

Ligation Procedure

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

1 µl of cloning vector
1 µl of 10x ligation buffer
0.5 µl of ATP (10 mM)
1–4 µl of *Pfu*-polished PCR product insert*
1 µl of *Srf*I restriction endonuclease (5 units)
1 µl of T4 DNA ligase (4 units)
ddH_2O to a final volume of 10 µl

**Note:* 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. A greater insert-to-vector ratio is necessary for unpolished inserts because there will be a decreased occurrence of PCR fragments with both ends blunted. It may be advantageous to optimize conditions for a particular insert using the following equation:

$$\text{pmole ends}/\mu\text{g of DNA} = 2 \times 10^6/\text{number of bp}$$

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

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

4. Store the sample on ice until transformation into competent *E. coli.*

E. coli Transformation

Competent cells are very sensitive to even small variations in temperature and must be stored at –80°C. Repetitive freeze-thawing will result in a loss of efficiency and should be avoided. It is important to use FALCON 2059 tubes for the transformation procedure, as the critical incubation period during the heat-pulse step described below is calculated for the thickness and shape of the FALCON 2059 tube. Also, β-mercaptoethanol has been shown to increase transformation efficiencies two- to threefold. Upon transformation, there seems to be a defined "window" of highest efficiency resulting from the heat pulse. Optimal efficiencies are observed when cells are heat-pulsed for 45–60 seconds. Supercompetent cells can be purchased commercially that yield extremely high efficiencies upon transformation.

PROTOCOL

Transformation Guidelines

1. Thaw competent cells on ice.

2. Gently mix the cells by swirling. Aliquot 40 µl of cells into a prechilled 15-ml FALCON 2059 tube.

3. Add β-mercaptoethanol (for a final 25 mM concentration) to the 40 µl of bacteria.

4. Swirl gently. Place on ice for 10 minutes; swirl gently every 2 minutes.

5. Add 2 µl of DNA from the heat-treated ligation reaction (step 4, Cloning Procedure).

6. Place on ice for 30 minutes.

7. Heat-pulse for 45 seconds in a 42°C water bath. The length of the heat pulse is critical for the highest efficiencies.

8. Place the transformation mixture on ice for 2 minutes.

9. Add 450 µl of preheated (42°C) SOC medium and incubate with shaking at 225–250 rpm for 1 hour at 37°C.

10. Plate 50–200 µl of the transformation mixture (100 µl is standard) using a sterile spreader to place the mixture onto the appropriate antibiotic-containing agar plates (a chromogenic substrate may be added to the LB plates to detect recombinants; see also β-Galactosidase Color Selection, below).

    Note: If plating ≥100 ml, the cells can be spread directly onto the plates. If plating <100 µl of the transformation mixture, increase the volume of the transformation mixture to be plated to a total volume of 200 µl using SOC medium.

11. Incubate the plates overnight (≥16 hours) at 37°C.

12. Choose white colonies for examination, avoiding colonies with a light-blue appearance or colonies with a blue center.

    Note: Colonies containing inserts that were initially white may turn very light blue after 2–5 days on the plate.

β-Galactosidase Color Selection

Phenotypic selection by disruption of the β-galactosidase (βGal) gene is often used to detect recombinants (Maniatis et al. 1982). Such phenotypic selection is monitored by the appearance of recombinants as white colonies and nonrecombinants as blue transformant colonies on XGal-containing agar plates. IPTG is often used as an inducer in conjuction with XGal.

PROTOCOL

Phenotypic Color Selection

1. Prepare a 100 mg/ml solution of XGal in dimethylformamide (DMF).

2. Prepare a 100 mM solution of IPTG in sterile, ddH_2O.

   Note: The XGal and IPTG solutions can be spread directly onto antibiotic-containing plates. Avoid mixing XGal and IPTG, as these chemicals will precipitate.

3. Add a 20-µl aliquot each of XGal and IPTG solutions onto agar plates. Spread immediately in an evenly distributed manner (a slight precipitate may be apparent).

4. Allow the plates to dry for 15–30 minutes before spreading transformants.

REAGENTS

Analysis of Cloned Recombinants

Recombinant screening kit
- ScreenTest recombinant screening kit (Stratagene, cat. no. 301800)

DNA minipreparation kits:
- ClearCut miniprep kit (Stratagene, cat. no. 400732)
- QIAGEN Plasmid Mini kit (QIAGEN, cat. no. 12123)
- Wizard Minipreps (Promega, cat. no. A7100)

DNA sequencing kits:
- Cyclist exo$^-$ *Pfu* DNA Sequencing kit (Stratagene, cat. no. 200326)
- Sequenase Version 2.0 DNA Sequencing Kit (USB/Amersham, cat. no. 70770)

Reagents required:
- Synthetic oligonucleotide primers
- Deoxynucleotide triphosphate mix (dNTP; 100 mM)
- PCR optimization buffers
  - Opti-Prime PCR optimization kit (Stratagene, cat. no. 200422)
  - The PCR Optimizer (Invitrogen, cat. no. K1220-01)
- Thermostable DNA polymerases (5–10 units)
  - *Taq* DNA polymerase
  - native *Pfu* DNA polymerase (Stratagene, cat. no. 600135)
  - cloned *Pfu* DNA polymerase (Stratagene, cat. no. 600153)
- *Taq* Extender PCR additive (Stratagene, cat. no. 600148)
- Ethidium bromide
- DNA sequencing primers
- 10x Cycle sequencing buffer (200 mM Tris-HCl, pH 8.8, 100 mM KCl, 200 mM $MgSO_4$, 100 µM $(NH_4)_2SO_4$, 1% Triton, 1 mg/ml BSA, 20 µM dATP, 50 µM dCTP, 50 µM dGTP, 50 µM dTTP)
- Stop dye mix (80% formamide, 50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol)
- Mineral oil (Sigma, cat. no. M-3516)
- Sterile toothpicks

Rapid Recombinant Screening Analysis by Colony-PCR

Recombinant insert analysis of colonies resulting from transformed cells can be performed in 1 day using colony-PCR (Costa and Weiner 1994e,f). Recombinant PCR screening allows the rapid and efficient detection of cloned inserts from most ColE1-based plasmids. By using primers asymmetrically distanced from the clonal insertion site, it is possible to discern both insert presence and orientation from the resulting PCR product (see Fig. 5). One can also conduct PCR using a triple primer set containing the two asymmetric primers and an additional, fragment-specific primer from the set used to generate the original fragment. Agarose gel analysis of the PCR using such a three-primer set confirms both the presence and the orientation of the cloned insert without the need for further restriction enzyme digestion.

Further characterization of the cloned inserts can be done using restriction enzyme analysis of the colony-PCR product. Restriction enzyme digestion of the recombinant-screen PCR products can be performed directly from the amplification reaction (Costa and Weiner 1994f). In addition to restriction analysis, the recombinant-screen PCR products can be further characterized by cycle sequencing (Hedden et al. 1992; Costa and Weiner 1994f; Kretz et al. 1994). Because the

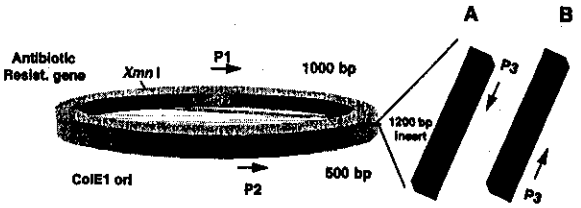

Figure 5 Recombinant screening method. Shown on the plasmid map are the PCR primers (P1 and P2) used in a colony-PCR screening procedure. PCR products produced using the colony-PCR method are analyzed by agarose gel electrophoresis. In a representative experiment in which a 1.2-kb insert is cloned into a plasmid, colonies selected directly from the transformation plates are inoculated into colony-PCR reaction mixtures, and PCR is conducted followed by agarose gel analysis. Nonrecombinants, which do not contain the insert, exhibit a 1.5-kb PCR product. Recombinants, which do contain the 1.2-kb insert, exhibit a 2.7-kb PCR product. Directionality of the cloned insert can be determined in a separate reaction by adding a third, insert-specific primer (P3) to the colony-PCR reaction mixture. Bidirectional cloning produces recombinants that contain inserts cloned in both directions, and the use of a third primer in the reaction mixture confirms the orientation of the cloned fragment (orientation A or B). An example of the recombinant screening of clones with a 1.2-kb insert by colony-PCR in the presence of a third primer indicates the orientation of the cloned insert after agarose gel analysis with a constitutive 2.7-kb PCR product and either a 1.7-kb PCR product (orientation A) or a 2.2-kb PCR product (orientation B).

primer set is designed to flank the polylinker by a distance of ≥500 bases on either side of the multiple cloning site, there is a retention of common priming sites used for DNA sequencing. Colony-PCR procedures that result in a single product (no spurious bands or primer-dimers) may be diluted and used in a cycle-sequencing reaction. High-resolution sequences have been consistently obtained using an aliquot of a 1:50 dilution of ScreenTest PCR products.

PCR-mediated clonal analysis allows one to screen numerous clones in a simple, rapid, and highly efficient manner. The procedure for recombinant screening by colony-PCR is outlined below. The recombinant screening protocol is outlined in four sections: preparation of the colony-PCR mixture; PCR parameters; analysis of the PCR results; and troubleshooting. It may be beneficial to evaluate the considerations in the troubleshooting section as a precautionary measure before proceeding with the PCR-based screening protocol. Then further experimentation and characterization of the PCR-generated products by restriction endonuclease analysis and DNA cycle sequencing are addressed.

PROTOCOL

Preparation of the Colony-PCR Mixture

As a positive control for this method, use nonrecombinant DNA (vector that does not contain insert). This will provide negative internal control colony-PCR. However, one may also transform the nonrecombinant, vector DNA and inoculate colonies from the transformation plate into the standard reaction mixture to serve as a positive control for the colony-PCR.

According to the number of reactions or multiples of reactions needed, prepare the PCR cocktail master mix in a single microcentrifuge tube *on ice* by adding the components *in the order indicated* below. After preparing the PCR cocktail master mix, follow the suggested guidelines outlined below.

Colony-PCR master mix

For the *PCR cocktail master mix:*

Sterile ddH_2O	40.6 µl
10X ScreenTest buffer	5 µl
dNTP mix (25 mM of each dNTP)	0.4 µl
Recombinant screening primer set (100 ng/µl)	2 µl
Taq Extender PCR additive (5 units/µl)	0.5 µl
Taq DNA polymerase (5 units/µl)	0.5 µl
Total reaction volume	49.0 µl

For the *control reaction:*

Nonrecombinant DNA (no insert; 1–5 ng/ml)	1 µl

For *insert orientation:*
Third, insert-specific primer (100 ng/μl) 1 μl

For *recombinant screening:*
Single colony toothpick inoculation

1. Aliquot 49 μl of the PCR cocktail master mix into each microcentrifuge tube on ice.

2. For control reaction(s), add 1 μl of the nonrecombinant DNA.

3. *Optional:* For insert orientation reaction(s), add a third, insert-specific primer to the appropriate reaction tubes.

4. For recombinant screening, stab the transformed colonies with a sterile toothpick and swirl the colony material into the appropriate reaction tubes. Immediately following inoculation into each reaction mixture, remove the toothpick and score onto antibiotic-containing patch plates for future reference.

   Note:
   - PCR inhibition may result in the event of excess colony material in the colony-PCR. It is important to note that only a small amount of colony material is necessary to perform the recombinant screening method. Stab the colonies from the transformation plate using the smaller ends of sterilized toothpicks. When performing the recombinant screening method from patch plates, only "touch" onto the patch and inoculate directly into the reaction tubes.
   - Toothpicks have the ability to "wick" liquid, so it is important to remove the toothpick immediately after inoculation.
   - Archive the screened colony material by using the inoculation toothpick to score an antibiotic-containing LB-agar plate.
   - See also Troubleshooting for PCR-mediated recombinant screening, below.

5. Mix each reaction gently.

6. Overlay each reaction with 30 μl of mineral oil.

7. Perform PCR using the recommended cycling parameters.

PCR Parameters

Depending on the needs of the investigator and the performance characteristics of the thermal cycler, sensitivity can be altered by changing both the number of cycles and the annealing temperature of segment 2. Also, depending on the oligonucleotide primer that is used, it may be advantageous to calculate its optimal annealing temperature. Several of the oligonucleotide primer selection programs listed in the Appendices (see pp. 681–686) accurately calculate the melting

temperature. Once a melting temperature is determined, a revised segment 2 can be constructed. Alternatively, this equation, which is valid for oligonucleotides shorter than 20 bases, and overestimates the melting temperature, can be used.

$$T_m = 2^oC\ (A+T) + 4^oC\ (G+C)$$

The following PCR program has been successfully used with 30-base oligonucleotide primers. The sensitivity of the program is determined by segment 2 and may need to be reoptimized when using a third, insert-specific oligonucleotide primer that is <30 bases.

Segment	Cycles	Time	Temperature
1	1	4 minutes	94°C
		2 minutes	50°C
		2 minutes	72°C
2	30	1 minute	94°C
		2 minutes	56°C
		1 minute	72°C
3	1	5 minutes	72°C

Analysis of PCR Results

The PCR products are analyzed using standard agarose gel electrophoresis. It is recommended to use a 1.0–1.5% (w/v) agarose gel for optimal resolution of the expected 500- to 6000-bp PCR products. Typically, 15 µl of each PCR is analyzed utilizing ethidium bromide staining. Images may be archived using conventional instant photography or computer-based imaging software.

TROUBLESHOOTING

Under optimal conditions, colony-PCR provides an adequate amount of DNA template that will yield maximum signal in the PCR. Undoubtedly, there will be variations in thermal cyclers and reagents that may contribute to signal differences in the experiments. The following are guidelines for troubleshooting these variations in PCR-mediated recombinant screening.

- *Low signal with control DNA.* Suboptimal reagents (e.g., *Taq* DNA polymerase) and/or the thermal cycler used in conducting the assay may account for the results. The positive control is a good indicator of amplification efficiency and, when using 1–5 ng of DNA according to the specified guidelines, has been calculated to yield amounts of PCR product approaching plateau levels.

- *Low signal in the screening samples.* PCR inhibition may result in the event of excess colony material in the colony-PCR. It is impor-

tant to note that only a small amount of colony material is necessary to perform the recombinant screening method. Stab the colonies from the transformation plate using the smaller ends of sterilized toothpicks. When perfoming the recombinant screening method from patch plates, only "touch" onto the patch and inoculate directly into the reaction tubes.

- *Loss of sample volume.* Reduced sample volume results when toothpicks are left in the reaction mixtures. Toothpicks "wick" the solution out of the reaction tubes, and for this reason, removal of the toothpicks shortly after the PCR cocktail master mix inoculation is strongly recommended.

- *Excessive signals in the samples.* This PCR-mediated screening method has been optimized on thermal cyclers whose temperature profiles are very exact and reproducible. Thermal cyclers whose transition times are very long inadvertantly add time to the PCR program and may result in excessive signals in both test samples and controls. In an attempt to reduce the signal, it may be advantageous to reoptimize segment 2 in the PCR program.

- *Multiple banding patterns.* This screening method has been designed with parameters optimized for use in colony-PCR where limited amounts of colony material are present. In the schematic representation of the recombinant screening method (see Fig. 5), two potential PCR products can be produced in the presence of a third, insert-specific oligonucleotide primer (P3). One PCR product is generated by P3+P1 or by P3+P2, and a second constitutive PCR product is generated by P1+P2. The method relies on the fact that, when limited amounts of template DNA are available, the smallest PCR product will be preferentially amplified. In cases in which pure, miniprep, or cesium-banded DNA is used, such purified DNA provides an optimally accessible template in cyclic amplification procedures, thereby producing both "expected" PCR products. Therefore, it is very important to calculate the expected PCR products when using a third, insert-specific oligonucleotide primer in directionality studies for the determination of insert orientation.

PROTOCOLS

Restriction Analysis of the PCR-generated Products

Upon deciphering insert presence, further characterization—such as the orientation of cloned inserts—can be achieved by restriction enzyme analysis. Restriction endonuclease digestion of the PCR-generated products can be performed directly from the amplification reaction.

1. Aliquot the following into a restriction enzyme digestion reaction as outlined below:

   5 µl of colony-PCR product
   2 µl of 10x enzyme-compatible buffer
   10–15 units of restriction endonuclease
   ddH_2O to a final volume of 20 µl

2. Incubate digestion at the recommended enzyme-specific temperature for 30–60 minutes.

3. Following incubation, load 10 µl of the digestion onto a 1.0–1.5% (w/v) agarose gel and analyze the PCR restriction digestion products by ethidium bromide staining.

DNA Cycle Sequencing of the PCR-generated Products

The PCR products from the recombinant screening procedure may be ultimately characterized by DNA cycle sequencing. Use of the Cyclist Exo⁻ *Pfu* DNA sequencing system (Stratagene) in combination with a radioactive label (e.g., α-^{33}P) provides high-resolution sequencing of the recombinant screening PCR products. Using the standard cycle-sequencing guidelines, PCR products may be diluted for use as follows:

1. Add 3 µl of the appropriate ddNTP to each of four termination tubes. Cap the tubes and keep on ice.

2. Prepare a 1:50 dilution of the recombinant screening PCR products into TE buffer.

3. For each PCR-generated template, combine the following reaction components *on ice.*

   per cycle-sequencing reaction:
   10 µl of diluted recombinant screen PCR product (~200 fmoles)
   1 µl of sequencing primer (~1 pmole*)
   4 µl of 10x cycle-sequencing buffer
   1 µl of [α-^{33}P]dATP (10 µCi)
   1 µl of Exo⁻ *Pfu* DNA polymerase (2.5 units)

   *Weight of DNA equal to 1 pmole:
   0.33 x N = ng of ssDNA, where N = number of bases in primer

4. In a separate microcentrifuge tube mix together:

   13 µl of ddH_2O
   4 µl of dimethylsulfoxide (DMSO)

5. Add the 17 µl of ddH_2O-DMSO mixture to the above cycle sequencing reaction solution to yield a final volume of 30 µl.

6. Aliquot 7 µl of the cycle-sequencing reaction mixture from step 5 into each of the four termination tubes already containing 3 µl of ddNTP. Mix thoroughly, making sure the reaction mix and the dideoxynucleotide mix are at the bottom of the tube.

7. Overlay the reactions with 20 µl of mineral oil.

8. Cycle the sequence reaction through an appropriate temperature profile. Optimum cycling parameters will vary depending on the template and primer, as well as the type of machine used. A useful PCR cycling profile is given below.

Segment	Cycles	Time	Temperature
1	30	30 seconds	95°C
		30 seconds	60°C
		60 seconds	72°C

9. Add 5 µl of stop mix below the mineral overlay and mix by pipetting.

10. Heat-denature the samples for 2–5 minutes at 80°C, then immediately load 2–4 µl of the samples onto a sequencing gel.

11. Using standard procedures, dry the gel and expose to autoradiograph film.

TROUBLESHOOTING

- *Little or no sample after cycling.* This may be due to sample evaporation, possibly resulting from insufficient mineral oil overlay. Add at least 20 µl of mineral oil and briefly centrifuge before cycling.

- *Faint bands or blank film.* (1) No radioactivity was added, or the radiolabel is old. (2) One of the reaction components is missing, or the reaction components were not thoroughly mixed. (3) The primer did not anneal efficiently. Reduce annealing temperature or redesign the primer. (4) Not enough template was used. (5) The template DNA is contaminated. Make sure excess salt and EDTA have not contaminated the preparation. The PCR product may need to be precipitated prior to template dilution (see Optional PCR Product Purification, above).

- *High background on the sequencing gel.* Too much template DNA. This can be a serious problem with short PCR products. Titrate down the amount of DNA added to the reaction.

- *Bands in multiple lanes.* (1) The primer annealed at multiple sites. A higher annealing temperature may help. (2) There are multiple templates in the sequencing reaction. PCR products containing multiple sequences or primer-dimer artifacts are present and one of the PCR primers was used as a sequencing primer. Gel purification should alleviate the problem. (3) There are gel compression artifacts. Increase the gel temperature (up to 60°C) or add formamide to the gel. (4) The template DNA is contaminated.

- *Blurry or smeared bands.* (1) Samples were not fully denatured prior to gel electrophoresis. (2) Old or improperly prepared acrylamide solutions were used. (3) Bad DMSO was used. Do not freeze-thaw DMSO more than once.

Traditional DNA Minipreparation and Restriction Enzyme Analysis

Alternatively, one could conduct routine plasmid DNA isolation after overnight incubation and determine both the insert size and orientation following restriction enzyme digestion and agarose gel analysis. A number of commercially available kits can be used that produce high-quality miniprep plasmid DNA (see Reagents: DNA minipreparation kits, above). Non-cycle procedures for DNA sequencing can be used from these minipreparations (Sequenase, USB).

CONCLUSION

PCR has both simplified and accelerated the process for cloning DNA fragments. It is now possible to synthesize primers and perform the PCR, cloning, and transformation reactions in a single day. The analysis of putative clones by colony-PCR and cycle sequencing can be completed the following day. The methods presented allow PCR cloning operations to exhibit more than 50% recombinant efficiency and facilitate PCR screening methods that can be completed in a rapid and highly efficient manner.

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