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# Amplification of Flanking Regions: New Applications and Performance Optimization of Single Specific Primer PCR and T-Vector Cloning

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*Single specific primer PCR[(a)] (SSP-PCR), designed for DNA amplification when only one gene-specific primer is available, has been shown to amplify fragments of up to 1.8kb. By optimizing this technique, we have been able to amplify significantly longer fragments. We have utilized this strategy in a variety of cloning applications. Here, we describe optimization of the SSP-PCR technique for the generation of long, specific amplification products.*

## Introduction

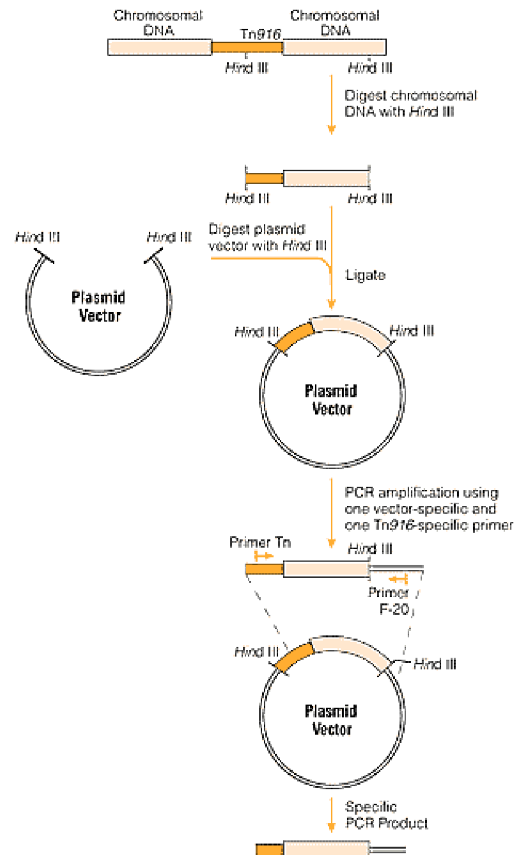
The need to clone genes encoding proteins of interest or regions contiguous to known loci has been a driving force for developing new techniques in molecular biology (1,2). Developments in PCR (3) in the last several years have circumvented many experimental obstacles by *in vitro* amplification of loci that are otherwise difficult to clone or study *in vivo*. One of the usual requirements for amplification is that the sequence at both ends of the desired locus is known, allowing synthesis of two inward facing primers. However, for many applications it is necessary to amplify a target DNA when sequence information for the design of only one primer is available. Inverse PCR, panhandle PCR and other anchored techniques have been developed to facilitate amplification of unknown DNA sequences (4-8). One of these techniques, single specific primer PCR (SSP-PCR; 8,9), has been used for amplifying fragments of up to 1.8kb (10). By optimizing this technique, we have been able to amplify fragments of up to 3.2Kb.

We have applied SSP-PCR to solve a variety of problems, including cloning of DNA contiguous to the site of insertion of the Tn*916* transposon in streptococci and cloning of 5´-regions of genes encoding three different streptococcal proteins (11-14). We have also developed a new application for cloning a locus encoding structural and biosynthetic genes for biosynthesis of mutacin II, a novel antimicrobial peptide from mutans streptococci (14). The procedure is based upon SSP-PCR amplification using restricted chromosomal DNA ligated into a suitable vector (8). We used two primers in the PCR -- one outward specific to the desired locus, and the second annealing to the vector pUC19. The specific amplicons were successfully cloned and sequenced to confirm that the amplified DNA is contiguous to the desired locus. Here, we report optimization of the SSP-PCR technique for the generation of long, specific amplification products.

*[(a)]PCR is a patented process. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process.*

## SSP-PCR

An example of the SSP-PCR protocol is illustrated in Figure 1. Chromosomal DNA is isolated from a strain harboring a transposon (e.g., Tn*916*) within the locus of interest. The chromosomal DNA is digested with a restriction endonuclease and ligated into linearized plasmid DNA. The resulting population of ligated molecules is comprised of a complex mixture of chromosomal DNA and chromosomal-plasmid DNA hybrids. The plasmid derived region of the hybrid molecules provides the downstream priming site (the anchor) for PCR amplification. The upstream primer may be specific for the transposon, or a gene-specific primer if sufficient sequence




information is available for primer design.

**Figure 1. Schematic illustration of single specific primer PCR.** Chromosomal DNA containing a Tn*916* insertion in a streptococcal strain is digested with a restriction endonuclease (e.g., *Hin*d III) and the restricted DNA is ligated into a plasmid vector digested with the same restriction enzyme. Two primers (Tn*916*-specific and vector-specific) are used to amplify a specific PCR product consisting of a fragment of chromosomal DNA surrounded by short Tn*916* and vector sequences.

The SSP-PCR protocol was successfully used with a transposon-specific primer to amplify a 0.85kb fragment adjacent to a Tn*916* insertion in *Streptococcus mutans* (Figure 2). No product was observed with the parental strain lacking a Tn*916* insertion.

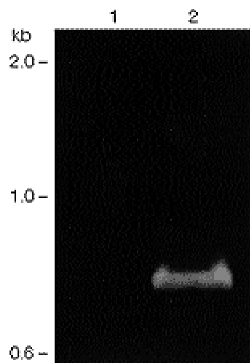



**Figure 2. Amplification of a DNA fragment contiguous to the Tn*916* insertion in *Streptococcus mutans*.** Chromosomal DNA samples (1µg) were digested with *Hin*d III. Aliquots (100-200ng) of the linearized chromosomal DNA were ligated into 60-120ng of linearized pUC19 vector DNA in a total volume of 25µl. After overnight ligation at room temperature, a 1µl aliquot was used for PCR amplification. Hot start PCR was performed using AmpliWax® PCR Gem 100 beads (19) in a total volume of 50-100µl. The optimized PCR reaction mixture contained 20-50pmol of the transposon-specific primer

TnLO-2 (20), 20pmol of the F-20 pUC19-specific primer, 20mM Tris-HCl (pH 8.4), 50mM KCl and 2.5mM $MgCl_2$. The mixture was pre-incubated at 80°C for 5 minutes before 2.5u of *Taq* DNA polymerase (diluted with 15µl of reaction buffer) was layered over the solidified wax barrier. The samples were denatured at 94°C for 2 minutes, followed by 35 cycles of 94°C, 30 seconds; 58°C, 30 seconds; 72°C, 90 seconds in a GeneAmp® PCR System 9600 thermal cycler (Roche Molecular Systems, Inc.). After a final incubation at 72°C for 10 minutes, the samples were stored at 4°C until used. A 15µl aliquot of each PCR reaction was separated on a 1.2% agarose gel, stained with ethidium bromide and photographed under UV illumination. Lanes: lane 1, a negative control containing DNA isolated from a parental strain without the Tn*916* insertion; lane 2, an 850 base pair PCR product amplified from chromosomal DNA isolated from Tn*916* mutant UA620-9.

# Optimization of SSP-PCR

This amplification technique requires one gene-specific primer, a ligation mixture of chromosomal DNA ligated into a plasmid vector (we used pUC19) and a second primer annealing to the vector. These components can be optimized prior to the amplification reaction. Previous reports suggest the vector:DNA ratio is critical for specificity of product and that double restriction enzyme cleavage is desirable (10). We found that single enzyme digests with slightly smaller amounts of vector improved the results. Furthermore, we found that use of the PCR hot start technique (15) significantly lowers the background.

Optimization of the concentrations of magnesium, nucleotides, template DNA, primers, annealing temperatures, number of cycles and other interdependent variables are important for yield, length of product and specificity in PCR. Interestingly, asymmetric ratios of gene-specific and vector-specific primers reduce the background of nonspecific amplification. The two most important factors, however, are the annealing temperature and time of extension.

In general, when significant background or undesirable products are detected, two rounds of SSP-PCR are recommended. A short first-round amplification (15 cycles) using a gene-specific primer and a vector-specific primer provides a template for the second round of PCR, which uses a nested vector-specific primer (i.e., closer to the multiple cloning site than the first primer) and the same gene-specific primer. Also, the use of larger volumes (100µl) and the hot start technique facilitate the amplification of specific products (data not shown).

Application of SSP-PCR in various chromosome-walking experiments in our laboratory has allowed amplification of fragments up to 3.2kb. The advantages of using this technique are illustrated in Figure 3, which shows the amplification of the 5´-portion of group B streptococcal oligopeptidase (13).

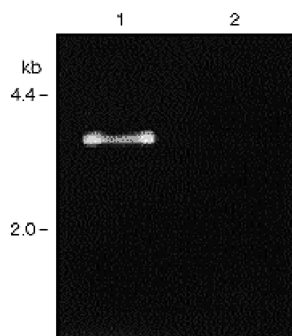



**Figure 3. Amplification of the 5´-portion of group B streptococcal oligopeptidase (13).** Chromosomal DNA samples (1µg) were digested with *Eco*R I. Aliquots (100-200ng) of the linearized chromosomal DNA were ligated into 60-120ng of *Eco*R I-linearized pUC19 vector DNA as described in Figure 2. Samples were amplified and subjected to agarose gel electrophoresis as described in Figure 2, except a gene-specific primer (primer 3; reference 13) was substituted for the TnLO-2 primer. Lanes: lane 1, a 3.2kb pair oligopeptidase PCR product amplified from ligated chromosomal DNA; lane 2, a negative control where the gene-specific primer was omitted from the amplification reaction.

# Cloning of SSP-PCR products

Specific PCR products can be sequenced directly or cloned using a T-vector system. We have successfully used Promega's pGEM®(b)-T and pGEM®-T(c) Easy Vector Systems. These vectors contain 3´-T overhangs that are complementary to the single deoxyadenosine residues that many of the polymerases used for PCR add to the 3´-ends of the amplification products. After overnight ligation at 14°C, the ligated DNA is transformed into high-efficiency (>$10^8$cfu/µg) competent cells (e.g., *E. coli* DH5alpha® (LTI) or JM109). If the yield of transformants or percentage of recombinants is lower than expected (we generally obtain recombinants in 6-10 of 10 white colonies) we recommend using *E. coli* Mri80, or a similar strain that lowers the vector copy number (16,17). This usually overcomes problems with growth and stability of clones that would otherwise be toxic in *E. coli*.

[(b)] *U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.*

[(c)] *Licensed under one or both of U.S. Pat. No. 5,487,993 and European Pat. No. 0 550 693.*

# Summary


The application of SSP-PCR technology allows amplification of unknown contiguous DNA fragments and facilitates cloning of genes encoding isolated proteins based on primers designed with available amino acid sequences. The PCR amplicons can be cloned in T-vector systems and the chimeric DNA amplified in standard *E. coli* strains (e.g., DH5alpha® or JM109) or in Mri80. The described PCR technique, in combination with long PCR (up to 35kb; 18), should provide significant benefits to studies employing chromosomal-walking and reverse genetics.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## Ordering Information

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| pGEM®-T Easy Vector System II | 20 reactions | A1380 |
| pGEM®-T Vector System I | 20 reactions | A3600 |
| pGEM®-T Vector System II | 20 reactions | A3610 |

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