Long Inverse PCR Using iProof™ Polymerase

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Introduction

Viruses have long been used as model systems to probe fundamental questions in molecular biology. The use of viruses to this end dates back to the 1930s, when the study of the T4 bacteriophage led to, among other things, the elucidation of the function of messenger RNA and the deciphering of the genetic code (Mathews et al. 1983). Using viruses as models for molecular study remains important today as we strive to understand new systems, tackle emerging diseases, and develop new tactics to fight pathogens.

Our laboratory's research focuses on the SSV1 virus. This UV-inducible virus was isolated from *Sulfolobus shibatae*, an acidic hyperthermophilic archaeon that lives in acidic sulfur springs with a pH near 3 and a temperature of around 80°C (Grogan et al. 1990). The 15.5 kb double-stranded circular DNA genome of the SSV1 virus contains several short repeated sequences and 34 open reading frames (ORFs), of which only four have known functions (Palm et al. 1991). The remaining ORFs show no similarity to any genes in public databases.

To investigate the function of the uncharacterized ORFs in SSV1, our laboratory has developed a method of long inverse PCR to quickly and effectively produce site-directed mutants. Inverse PCR was first described by Howard Ochman and colleagues (1988) and was designed to amplify regions of unsequenced DNA that flank regions of known sequence. In this technique, the DNA is first digested with a restriction enzyme, and the fragment containing the known sequence and flanking regions is ligated to form a circle. Next, using primers oriented outward from the area of known sequence, the rest of the fragment (i.e., the flanking regions) is amplified and can then be sequenced.

Unlike the method described by Ochman et al., the procedure we use amplifies the entire viral genome or slightly less (up to 20 kb). After amplification, the linear amplicon can be ligated together to produce a deletion mutant, the amplicon can be ligated to an insert to produce replacement mutants, or the entire genome can be amplified and ligated using primers containing mismatches to produce site-directed mutants. Transformation with these mutants produces a higher percentage of positive clones than transposon mutagenesis and other methods. This technique should be useful for rapidly producing site-directed mutations in other viruses with relatively large circular genomes, in plasmids, and in episomal DNA where other methods used to induce mutations prove ineffective.

In this report, we use iProof polymerase to amplify the entire 15.5 kb genome of the SSV1 virus from a shuttle vector consisting of the viral genome and an inserted bacterial plasmid. Further, we amplified the entire viral genome and replaced the original bacterial plasmid with one conferring resistance to a different antibiotic. Finally, this product was amplified from another site in the viral genome to remove a specific gene. As a result of the high fidelity of the iProof polymerase, both of these constructs show no detectable mutations and their ability to infect and reproduce in their host is similar to that of the wild-type virus.

Methods

Shuttle Vector Construction

A fusion between the bacterial plasmid pBluescript SK+ and the SSV1 virus was constructed as previously described by Stedman et al. (1999). Briefly, the 2,961 bp bacterial plasmid was inserted into a neutral site in the viral genome and was found to replicate similarly to the wild-type virus. Packaging this extra DNA seems to pose no problem for the virus since replication, stability, insertion, and virion structure are all comparable to wild type (Stedman et al. 1999). Shuttle vector genomes were purified from *E. coli* using alkaline lysis as described in Stedman et al. (1999).

Amplification

Primer design — To amplify the entire SSV1 genome from the original shuttle vector, standard M13 forward (–20) and M13 reverse (–27) primers were used with their sequences unchanged (Table 1). Primers for the second amplification (Del right and Del left), which used product from the first PCR as template, were designed to remove the complete gene from the virus and to allow the directional cloning of different genes. To this end, primers were designed so that their 5' ends flanked the ORF to be removed, overlapping the start codon. The length of the primer was extended in the 3' direction for approximately 25 bases, and extension was stopped when a GC clamp of at least one base was present (Table 1).

Table 1. Primers used in PCR. Bold letters show restriction sites, green letters indicate mispaired bases. Italics indicate the start codon of the removed gene.

Name	Sequence	T_m, °C
M13 forward (–20)	GTAAAACGACGGCCAGT	53.0
M13 reverse (–27)	CAGGAAACAGCTATGAC	47.3
Del right	CGTCTTATCTTTCGT***CAT***TTC**A**CCT**GGT**ACTATTATGG	58.3
Del left	GGGGTCTGACA**GGCG**C**C**GTATCACTATC	55.4

Primer sequences were checked for hairpins and other secondary structures using mfold software (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/; Zuker 2003), with Na^+ concentrations set at 50 mM and Mg^{2+} concentrations set at 0 mM. Primers were redesigned with different sequences if the T_m of the hairpin structure was within 15°C of the predicted T_m of the duplexed primer/template pair. Bases were modified to allow the insertion of restriction endonuclease cleavage sites for directional cloning (Table 1). Final T_m predictions were calculated with HyTher software (http://ozone2.chem.wayne.edu/hyther/hytherm1main.html), which predicts nucleic acid hybridization thermodynamics, taking into account mispairing. The T_m was adjusted by increasing or decreasing the 5' end of the primer to allow the predicted T_m values of forward and reverse primers to be between 55 and 60°C and within 3°C of each other.

PCR conditions — Amplification was carried out using the primers listed in Table 1 and the PCR reagents listed in Table 2, in a DNA Engine Dyad® thermal cycler equipped with a gradient block. Temperature calculations were estimated by the instrument and all reactions were carried out in 20 µl volumes.

Table 2. PCR parameters.

Reagent	M13 amplification	Del amplification
Buffer	1x HF buffer	1x HF buffer
dNTPs	0.2 mM/base	0.2 mM/base
Template	3 pM	6 pM
Forward primer	M13 F, 250 nM	Del right, 250 nM
Reverse primer	M13 R, 250 nM	Del left, 250 nM
Polymerase	0.02 U/µl iProof	0.02 U/µl iProof

Optimization of specific annealing temperatures is critical for decreasing nonspecific product production, especially with templates that contain repetitive elements such as the SSV1 genome. Therefore, temperature optimization was carried out for both primer sets. Figure 1 shows a temperature optimization with the M13 primers, starting at 3°C below the calculated annealing temperature of 53°C for standard PCR and increasing to above the optimal T_m. In most cases, the optimal temperature was 7–10°C higher than the calculated annealing temperature for standard PCR conditions.

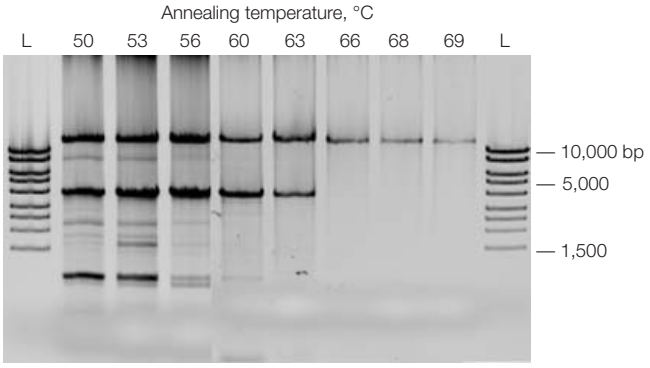

Fig. 1. Annealing temperature optimization. Long inverse PCR of the SSV1 shuttle vector was performed using varying annealing temperatures (indicated at top of gel). PCR products were run on an agarose gel to determine which was the lowest annealing temperature to prevent nonspecific amplification, in this case, 66°C. Lane L, MassRuler, high range DNA ladder (Fermentas).

The conditions used for amplification of each construct followed the manufacturer's guidelines. This included an initial denaturation step at 98°C for 3 min, followed by 30 cycles of 15 sec denaturation at 98°C, 15 sec annealing with the temperature optimized as described above (66°C for M13, 64°C for Del), and an 8 min extension at 72°C. A final 8 min extension was done at 72°C after the 30 cycles.

Ligation

Because iProof polymerase generates blunt-end DNA fragments during amplification, the M13 amplicon was cloned into the pCR Blunt II-TOPO vector (Invitrogen) following the manufacturer's instructions. This kit is designed to accept inserts that lack a 5' phosphate; therefore, no modification to the amplicon was necessary. The PCR product was gel-purified, and the gel containing the band was digested with β-agarase I (New England Biolabs). The product was then quantified relative to a standard by gel fluorescence, and 8 ng was added to one TOPO reaction kit as directed by the manufacturer.

Ligation of the deletion construct was carried out in a similar manner. The PCR product was circularized by adding 5' phosphates to the amplicon and ligating the blunt ends produced by iProof polymerase to each other. Gel-purified PCR product (500 ng) was added to a reaction of 1x T4 ligase buffer containing 1 mM ATP (New England Biolabs), 2 µl PEG 4000 (Sigma), and 10 U polynucleotide kinase (New England Biolabs) in a total volume of 40 µl. The reaction was incubated at 37°C for 1 hr, after which 20 U T4 ligase was added, and the reaction was incubated at 16°C for 4 hr.

Transformation Into *E. coli*

For the M13 construct, the entire ligation was transformed by heat shock into the StAble 3 strain of *E. coli* cells (Invitrogen). Transformation typically gave low yields (10^4 colonies/µg transformed). After 48 hr of growth, the smallest colonies were selected from the plates and grown in LB broth. Plasmids were purified from 5 ml liquid cultures by alkaline lysis. Preparations were screened for full-length constructs by restriction fragment length polymorphism (RFLP) analysis.

For the Del amplicon, a 10 µl aliquot of the reaction (125 ng) was transformed into chemically competent StAble 3 cells and plated as described above.

Transformation Into *Sulfolobus*

Shuttle vectors purified from *E. coli* were transformed by electroporation into the host *S. solfataricus* as described previously (Stedman et al. 2003).

Viral Production

Viral production of the amplified viral genomes was detected in three ways. First, transformed *S. solfataricus* was spotted onto lawns of uninfected *S. solfataricus,* and the cultures were examined for the presence of viral plaques. Second, RFLP analysis of purified shuttle vector genomes from infected strains was used to detect the presence of reproducing virus. Finally, PCR was used to amplify the area surrounding the removed gene, and the PCR products were run on a gel to ascertain that bands of the correct size were produced in the different mutants. PCR conditions were as follows: initial denaturation at 95°C for 5 min, subsequent denaturation at 95°C for 15 sec, annealing at 52°C for 15 sec, and extension at 72°C for 1.5 min. After 30 cycles, a final extension at 72°C for 5 min was used.

Results and Discussion

Both the M13 amplification of the 15.5 kb viral genome and the subsequent 18.5 kb Del construct amplified from the PCR product of the M13 amplification yielded functional viruses upon transformation into the laboratory host *S. solfataricus*.

RFLP analysis (Figure 2) showed that, of the first five colonies screened, one contained the correct insert. This method required substantially less screening of colonies than other methods, such as transposon mutagenesis or partial restriction digestion and ligation (data not shown).

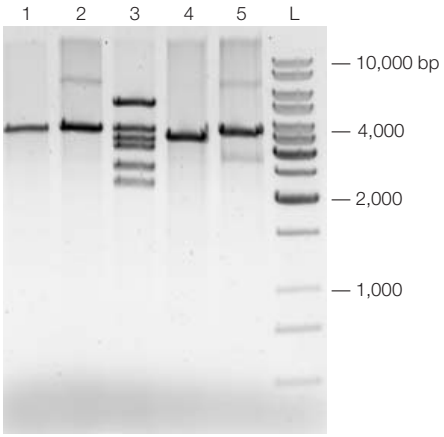

Fig. 2. RFLP screening of clones. DNA purified from *E. coli* transformed with the M13 PCR product was treated with EcoRI to determine whether the full-length clone was present. Of five transformations tested, only one, in lane 3, contained the 19.2 kb full-length clone. Lane L, 1 kb GeneRuler DNA ladder (Fermentas).

We used three different methods to verify that virus was being produced by the amplified viral genomes in *S. solfataricus*. First, viral plaques were seen after *S. solfataricus* transformed with the shuttle vector was spotted onto lawns of uninfected *S. solfataricus* (data not shown). Second, purification of shuttle vector genomes from infected strains and RFLP analysis showed the presence of reproducing virus (data not shown). Finally, PCR of the area surrounding the removed gene showed bands of correct size in the different mutants (Figure 3).

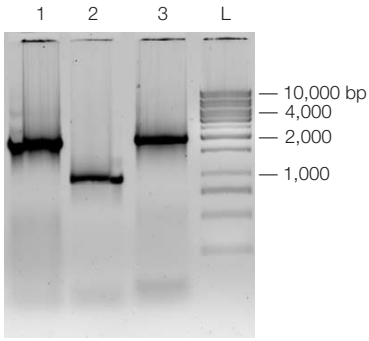

Fig. 3. Amplification of sequences surrounding the removed gene. Sequences were amplified by PCR and analyzed in a gel. Lane 1, M13 amplicon; lane 2, Del amplicon; lane 3 wild-type virus; lane L, 1 kb GeneRuler DNA ladder (Fermentas).

Proper amplification of the template required stricter adherence to specific reaction parameters, including template concentration, dNTP concentration, and reaction volume, than traditional PCR (data not shown). Template concentration had to be optimized for each primer set as well as for each DNA extract. It was often found that amplification was successful over only a narrow concentration range (less than an order of magnitude). The presence of varying amounts of contaminating proteins, sheared DNA, or both, in individual preparations and the sensitivity of the reaction may have led to varying amounts of template required for each preparation and to the necessity for individual optimization with each extract. Successful reactions occurred only at dNTP concentrations of 200 µM. Reaction volumes also affected the efficiency of the reaction, with the best results seen in small (20 µl) reactions.

We compared other high-fidelity polymerases in the same procedure, but none was effective at amplifying the template without producing smaller, nonspecific bands (data not shown). In addition to this, iProof polymerase had the fastest extension time of any of the high-fidelity polymerases, allowing completion of reactions in 8 hr as opposed to over 20 hr for other polymerases.

In summary, this method represents a rapid and efficient method for amplifying and mutating large plasmids and circular viral genomes.

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