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Rapid one-step recombinational cloning

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ABSTRACT

As an increasing number of genes and open reading frames of unknown function are discovered, expression of the encoded proteins is critical toward establishing function. Accordingly, there is an increased need for highly efficient, high-fidelity methods for directional cloning. Among the available methods, site-specific recombination-based cloning techniques, which eliminate the use of restriction endonucleases and ligase, have been widely used for high-throughput (HTP) procedures. We have developed a recombination cloning method, which uses truncated recombination sites to clone PCR products directly into destination/expression vectors, thereby bypassing the requirement for first producing an entry clone. Cloning efficiencies in excess of 80% are obtained providing a highly efficient method for directional HTP cloning.

INTRODUCTION

As a growing number of gene sequences become available, there is an increasing need for rapid cloning to determine gene function. To facilitate high-throughput (HTP) cloning the method of choice should be simple, efficient, and compatible with any DNA sequence to be cloned while providing directionality. Many methodologies have been developed for directional cloning (1–4). Among the available technologies, a powerful cloning method has been developed based on the well-characterized bacteriophage λ site-specific recombination system (1,5–8). Site-specific recombination is used for bacteriophage integration into the host genome during lysogeny and excision of the prophage following induction. The integration of phage λ takes place at a special attachment (*att*) site in the bacterial genome.The sequence of the *att* site in the bacterial genome is called *att*B (also called BOB′), whereas the sequence in the circular phage genome is called *att*P (also called POP′). The integration itself is a sequential exchange requiring both the phage Int protein and the bacterially encoded protein IHF (integration host factor). As a result of the integration two new hybrid sites are generated, *att*L (BOP′) and *att*R (POB), which flank the intervening phage DNA (prophage). During phage induction, the reaction goes in the opposite direction (excision) liberating the phage and recreating the *att*P and *att*B sites. This excision reaction is mediated by the Int, IHF and a second phage encoded protein, excisionase (Xis) (5–7).

A universal cloning technology referred to as Gateway® Cloning has been developed based on the λ site-specific recombination system (5). This cloning method provides a simple system for the transfer of DNA sequences between different cloning vectors, while maintaining both the open reading frame and orientation of the insert. The Gateway® cloning system is based on two recombination reactions, one is called the BP reaction (recombination between *att*B and *att*P sites) and the second is called the LR reaction (recombination between *att*L and *att*R sites). In both the BP and LR reactions, specific recombination sites are used for gene cloning and transfer from one vector to another. In the BP reaction, the PCR product (or cloned gene) is flanked by the site-specific recombination sites *att*B1 and *att*B2, which recombine with the *att*P1 and *att*P2 sites in the donor vector. This recombination is mediated by an enzyme mixture consisting of Int and IHF proteins. The resultant product is an entry vector containing the cloned sequence flanked by new recombination sites *att*L1 and *att*L2. To transfer the insert into an expression vector, the *att*L1 and *att*L2 sites recombine with the *att*R1 and *att*R2 sites, generating the newly formed recombination sites *att*B1 and *att*B2. This reaction is mediated by an enzyme mixture containing Int, IHF and Xis.

The Gateway® cloning system is based on a two-step process, where the DNA sequence is first cloned into the entry vector providing the template for the subsequent transfer into the destination vector(s). The transfer of DNA sequences into the destination vector is a rapid simple one-step reaction that saves considerable time and effort over traditional sub-cloning methods based on restriction enzymes and ligase. Since the system permits rapid transfer of DNA fragments into multiple destination vectors containing different regulatory elements and/or epitope tags, it readily supports different applications ranging from the functional analysis of genes to protein purification. On the other hand, for projects where the sequences are cloned into a single destination vector the requirement for cloning into the entry vector represents an unnecessary step. Accordingly, to meet our needs for

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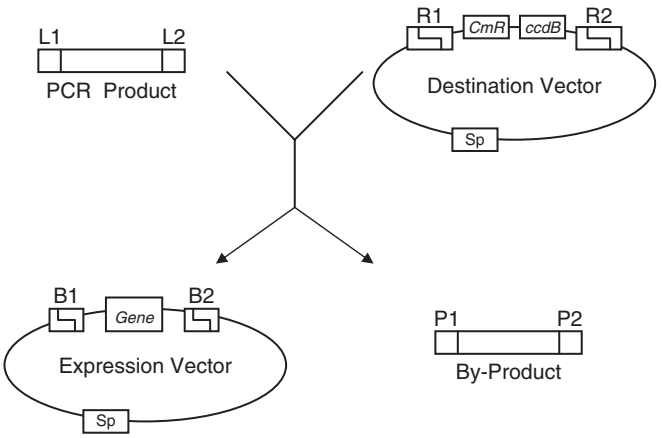

Figure 1. A schematic diagram of one-step recombination cloning. L1 and L2 refer to attL1 and attL2 sites. R1 and R2 refer to attR1 and attR2 sites. B1 and B2 refer to attB1 and attB2 sites, P1 and P2 refer to attP1 and attP2 sites.

a HTP single vector cloning system we have developed a one-step cloning method, which uses truncated *att*L recombination sites to clone PCR products directly into destination/expression vectors (Figure 1).

The *att*L sites used in the LR reaction are large (125 bp) and they share significant homology (95%) at the DNA level, both of which are problematic for PCR amplification. The decision to investigate the use of truncated *att*L sites rather than using the much smaller *att*B sites (25 bp) was based on the following considerations. The truncated *att*L sites are designed to be compatible with any of the existing Gateway® destination vectors, which harbor *att*R1 and *att*R2 sites. Conversion to a BP-based cloning system would require redesign of our existing Gateway® vectors that have been shown to provide good gene expression. Since the BP reaction generates *att*L1 and *att*L2 sites, the resultant vector would have >100 bp of extraneous sequence between the expression elements and the gene of interest. Finally, *att*L1 and *att*L2 contain several ATG codons that are likely to compromise translation of the desired coding sequence (9,10).

Our one-step LR recombination method has been successfully employed for the HTP cloning of hundreds of genes and fragments amplified from a variety of sources. Our cloning system is PCR based and cloning efficiencies of >80% are observed. The technology is amenable to HTP cloning and is a simple alternative to the Gateway® two-step cloning system.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Plasmids were introduced into *Escherichia coli by* either electroporation or by chemical transformation (2). The following *E. coli* strains were employed: DH10B [genotype: F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ *lacX74 deoR recA1 araD139* Δ (*ara leu*) 7697 *galU galK rpsL endA1 nupG*] and DH5α [genotype: F- *supE*44 Δ*lacU*169 *f80 lacZ*Δ M15 *hsdR17 recA*1 *′endA1 gyrA96 thi-1 relA1*] (Invitrogen, CA, USA). Cells were propagated in LB medium (Luria–Bertani) containing the appropriate antibiotic. Constructs harboring the *ccd*b gene were propagated in *E. coli* strain DB3.1 [genotype: F- *gyr*A462 *end*A1 Δ(*sr*1-*rec*A) *mcr*B *mrr hsd*S20(rB-, mB-) *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Smr) *xyl*5 Δ*leu mtl*1].

Construction of destination vectors

Two destination vectors were employed in this study. Both vectors contain the *att*R site for recombinational cloning, but use different antibiotic resistance markers, spectinomycin (Spec) or kanamycin (Kan), for selection in *E. coli*. The vectors also harbor the *ccdB* gene, which is a potent gyrase inhibitor and is toxic to *E. coli*. Plasmids containing *ccd*B gene are propagated in the *E. coli* strain DB3.1, which harbors a mutation in the gyrase gene (*gyr*A462) that confers resistance to the toxic affects of the CcdB protein (11,12).

Enzymes and reagents

Restriction enzymes were purchased from New England BioLabs (Beverly, MA, USA). Platinum Taq DNA Polymerase, High Fidelity (HiFi Taq) and high fidelity Herculase and Pfu turbo polymerases were purchased from Invitrogen and Stratagene (La Jolla, CA, USA), respectively. DNA and PCR product purification kits were purchased from Qiagen (Germany). Gateway® LR Clonase mix was purchased from Invitrogen. Most chemicals and antibiotics were purchased from Sigma (St Louis, MO, USA). Plastic ware including PCR plates, tubes and pipette tips were purchased through VWR.

PCR amplification and PCR product purification

Addition of the *att*L site-specific recombination sites to the ends of the DNA sequence to be cloned is achieved using a two-step PCR reaction. The first PCR is performed with gene-specific primers containing tails ranging from 9 to 14 nucleotides. The nucleotide tails provide the homology for addition of the *att*L sites in the second round of PCR [for example *att*L1 (SbfI)/*att*L2 (XhoI), *att*L1-T1/*att*L2-T1, and *att*L1-T2/*att*L2-T2]. In most cases, two types of gene-specific primers were designed and used in this study; Primer pair 1 consists of a 5′ oligonucleotide of the following sequence AGGCTcctgcaggACCATG $NNNNN_{15-25}$, where N corresponds to gene-specific sequence of a target gene downstream of the ATG initiation codon. The 5′ primer further contains an SbfI recognition sequence (cctgcagg) and a Kozak consensus sequence (ACCATG) (9,10). The sequence of 3′ oligonucleotide is as follows GAAAGCTGGGTctcgagCTAN $NNNN_{15-25}$, where N is the reverse complement of gene-specific sequence and ctcgag is an XhoI recognition sequence (Table 1). Primer pair 2 consists of the 5′ oligonucleotide GCTcctgcaggACCATG$NNNNN_{15-25}$ and a 3′ oligonucleotide CTGGGTctcgagCTANNN NN_{15-25} (Table 1). For both primer pairs, the underlined nucleotides correspond to the 'universal' tail. Additional primer pairs used in this study are listed in Table 1.

Several alternative primers were used to introduce the *att* sites in the 2nd PCR reaction. Primer pair 1 contains the 5′ *att*L1 (SbfI) oligonucleotide and the 3′oligonucleotide

Table 1. The DNA sequences of the tagged gene-specific primers and the universal primers used in this study

Primer	Sequence	Universal Tail
attL1(SbfI)	ccccAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACA AATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAG CAGGCTcctgcaggACCATG	AGGCTcctgcaggACCATGNNNNN$_{15–25}$ GCTcctgcaggACCATGNNNNN$_{15–25}$
attL2(SbfI)	ccccCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAAC AAATTGATAAGCAATGCTTTCTTATAATGCCAACTTTGTACAAGAAA GCTGGGTctcgagCTA	GAAAGCTGGGTctcgagCTANNNNN$_{15–25}$ CTGGGTctcgagCTANNNNN$_{15–25}$
attL1-T1	ccccGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCA GGCTcctgcaggACCATG	AGGCTcctgcaggACCATGNNNNN$_{15–25}$ GCTcctgcaggACCATGNNNNN$_{15–25}$
attL2-T1	ggggGATAAGCAATGCTTTCTTATAATGCCAACTTTGTACAAGAAAG CTGGGTctcgagCTA	GAAAGCTGGGTctcgagCTANNNNN$_{15–25}$ CTGGGTctcgagCTANNNNN$_{15–25}$
attL1-T2	ccccTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTcctgcaggACCATG	AGGCTcctgcaggACCATGNNNNN$_{15–25}$ GCTcctgcaggACCATGNNNNN$_{15–25}$
attL2-T2	ggggTCTTATAATGCCAACTTTGTACAAGAAAGCTGGGTctcgagCTA	GAAAGCTGGGTctcgagCTANNNNN$_{15–25}$ CTGGGTctcgagCTANNNNN$_{15–25}$
attL1-T3	ccccGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGT ACAAAAAAGCAGGCTcttaattaagACCATG	AGGCTcctgcaggACCATGNNNNN$_{15–25}$
attL2-T3	ggggGTTGCAACAAATTGATAAGCAATGCTTTCTTATAATGCCAACTTTGTA CAAGAAAGCTGGGTcctgcaggctcgagCTA	GGTcctgcaggctcgagCTANNNNN$_{15–25}$
attL1-T4	ccccGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAA TGCCAACTTTGTACAAAAAAGCAGGCTcttaattaagACCATG	AGGCTcctgcaggACCATGNNNNN$_{15–25}$
attL2-T4	ggggGTGACCTGTTCGTTGCAACAAATTGATAAGCAATGCTTTCTTATAAT GCCAACTTTGTACAAGAAAGCTGGGTcctgcaggctcgagCTA	GGTcctgcaggctcgagCTANNNNN$_{15–25}$

The N's represent the gene specific portion of the primer while the subscript 15–25 represents the length of the primer, which varies depending on the G + C content of the fragment/gene being amplified.

*att*L2 (XhoI) oligonucleotide. Primer pair 2 consists of a 5′ oligonucleotide *att*L1-T1 oligonucleotide and a 3′ *att*L2-T1 oligonucleotide. Pair 3 consists of a 5′ *att*L1-2 oligonucleotide and a 3′ *att*L2-T2 oligonucleotide. Pair 4 contains the 5′ oligonucleotide *att*L1-T3 and the 3′ oligonucleotide *att*L2-T3. Pair 5 contains the 5′ oligonucleotide *att*L1-T4 and 3′ oligonucleotide *att*L2-T4 (Table 1).

The first PCR reaction was performed using tailed gene-specific primers in a 25 µl total volume reaction containing 2.5 µl of 10× Herculase (or Pfu) PCR buffer, 0.5 µl of dNTPs (10 mM), 1 µl of cDNA or gDNA (~20 ng/µl), 0.5 µl of 5′ oligonucleotide (10 µM), 0.5 µl of 3′ oligonucleotide (10 µM), 0.2 µl of Herculase (5 U/µl) (or 0.5 µl of Pfu; 5 U/µl) and sterile water to 25 µl. For Platinum Taq Polymerase High Fidelity the reaction contained 2 mM $MgSO_4$. PCR was done for 15–17 cycles. The following cycling parameters were used for the first PCR reaction; 1st step: 95°C for 2 min; 2nd step: 95°C for 30 s; 3rd step: 55°C for 30 s; 4th step: 68°C for 6 min (1 to 1.5 min per kb insert); 5th step: 15 cycles total (from step 2 to 4); 6th step: 72°C for 5 min and 7th step: 10°C on hold.

The second PCR was performed with universal primers for 15–17 cycles in a 50 µl total volume reaction under the same conditions described earlier. All PCR reactions were carried out using a DNA Engine Tetrad (PTC-225) (MJ Research, MA, USA).

PCR products were size fractionated on 0.7–1.0% agarose gel and excised with a scalpel. PCR products were purified using the QIAquick PCR purification kit from Qiagen (Germany) according to the manufacturer's instructions. PCR products were eluted in either 30 µl of elution buffer (EB) provided by the manufacturer or 10 mM Tris–HCl, pH8.0. The sizes of the PCR products were confirmed by electrophoresis of 2–4 µl of the purified PCR products.

LR cloning reaction

The gel purified second round PCR products were used directly for LR cloning reaction. Each reaction contains 4 µl of LR Reaction buffer (5×), 3–8 µl of the second PCR product (~50–300 ng), 0.5 µl of destination vector (100–250 ng), 4 µl of Gateway® LR Clonase mix (Invitrogen). The reaction volumes were adjusted to 20 µl with TE. The reactions were incubated overnight at ambient temperature, unless otherwise stated.

A total of 0.5–1.0 µl of each LR reaction were mixed with 20 µl of ElectroMax DH10B competent cells (Invitrogen) on ice, and loaded into an electroporation cuvette (0.2 mm) (Bio-Rad, CA, USA or Invitrogen). Cells were electroporated at 1.8 kV using a BIO-RAD Micro Pulser (Bio-Rad). Electroporated cells were incubated with 80 µl of SOC medium (Invitrogen) at 37°C for 1 h, and plated onto LB agar plates containing either spectinomycin (100 µg/ml) or kanamycin (50 µg/ml). Plates were incubated overnight at 37°C.

Screening and sequencing

Eight colonies from each electroporation were picked into 96-well deep well plates (Qiagen) containing LB medium plus the appropriate antibiotics. Following inoculation, the plates were incubated overnight on a rotary shaker (250 r.p.m.) at 37°C. The overnight culture was used directly as a PCR template. Cultures were screened by PCR amplification with either gene-specific or universal primers (located on the backbone vector). The screening reactions contain the following: 2.5 µl of Taq High Fidelity PCR buffer (10×), 1.0 µl of $MgSO_4$ (50 mM), 0.5 µl of dNTPs (10 mM), 1 µl of cultured cells, 0.5 µl of 5′ oligonucleotide (10 µM), 0.5 µl of 3′ oligonucleotide (10 µM), 0.2 µl of Platinum Taq High Fidelity (5 U/µl) and 18.8 µl

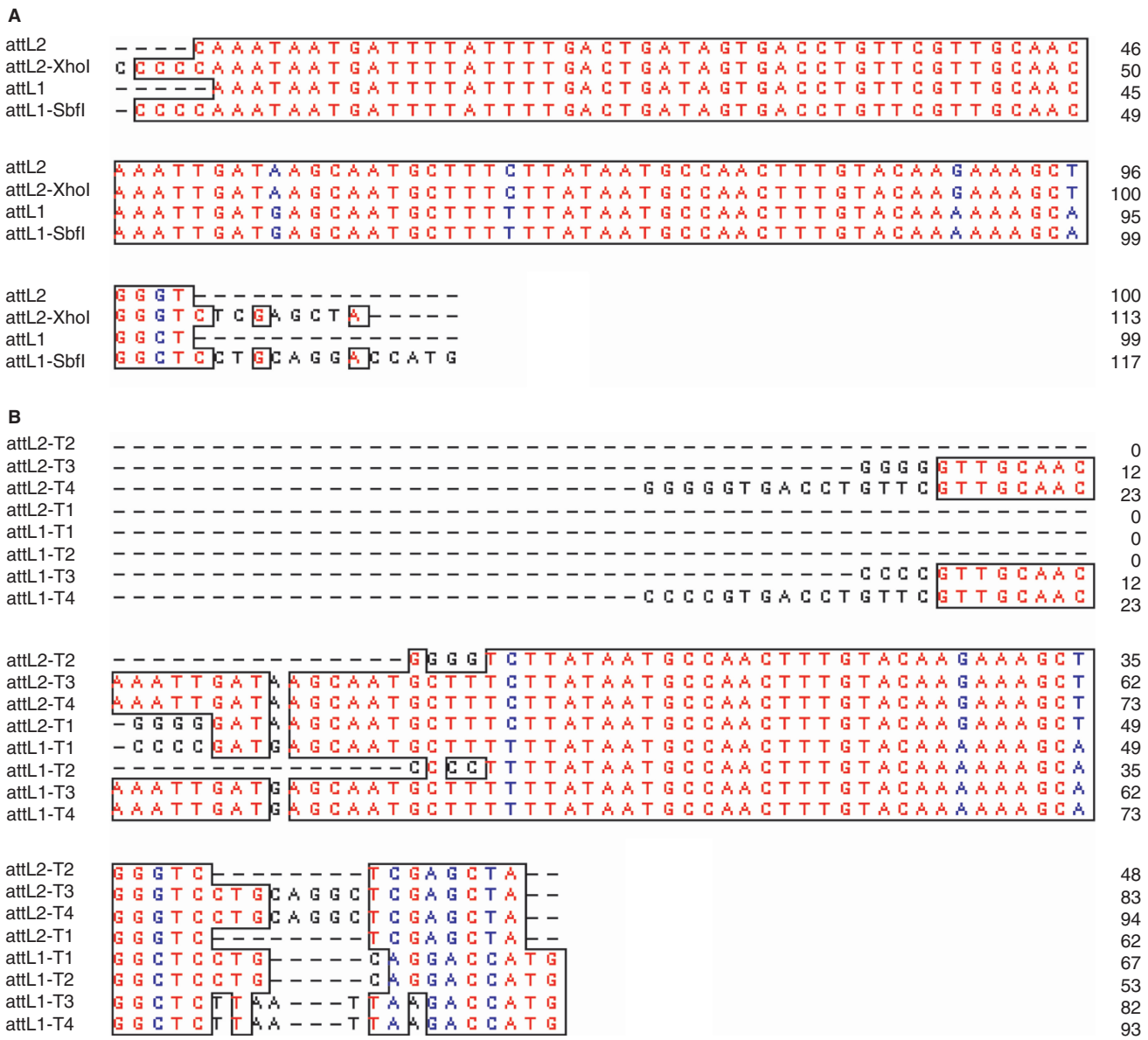

Figure 2. Alignment of attL sites (primer oligonucleotides). (**A**) Alignment of original attL1 (forward) and attL2 (reverse complementary) sites as well as modified attL1(SbfI) and attL2(XhoI) sites. (**B**) Alignment of modified attL1-T1 and attL2-T1, attL1-T2 and attL2-T2, attL1-T3 and attL2-T3, and attL1-T4 and attL2-T4 oligonucleotides.

of sterile water. The cultured-cell PCR was performed for 25 cycles with same PCR program as described earlier.

Plasmid DNA was isolated using a Qiagen DNA Isolation Kit according to the manufacture's instruction. Purified DNA was sequenced using an ABI3730 DNA Analyzer (Applied Biosystem, Foster City, CA, USA) and the sequence was assembled and analyzers using the DNAstar analysis software (DNASTAR Inc., Madison, WI, USA).

RESULTS

Primer design and two step PCR

To facilitate the directional cloning of large numbers of genes, we have developed a rapid one step cloning protocol based on the phage λ site-specific recombination system (1,6). The first step in the process was to establish a method to efficiently introduce the necessary recombination sites (*att*L sites) onto the ends of the DNA molecules to be cloned. One possibility was to simply tether the *att*L sequences to the gene-/sequence-specific primers and use these primers directly for PCR amplification. This is not practical since the *att*L sequences (*att*L1 and *att*L2) are relatively large (125 bp each) and are 95% identical at the nucleotide level. Figure 2 depicts the alignment of *att*L1 and *att*L2, as well as the truncated versions of the respective sites, which are discussed subsequently. The large size exceeds the current limits of cost efficient oligonucleotide synthesis, and even if synthesis were practical, the size coupled with the high identity is expected to negatively affect specificity while promoting

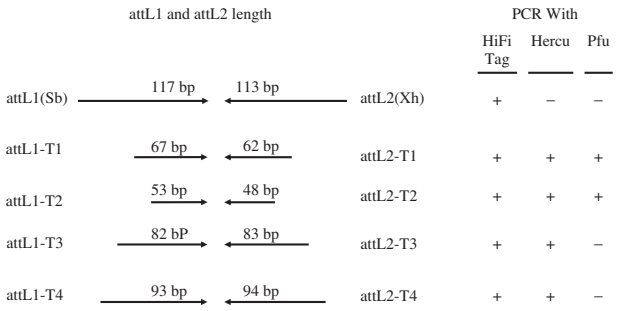

Figure 3. Comparison of the PCR results using truncated attL sites and different DNA polymerases. HiFi tag refers to high-fidelity tag polymerase. Hercu refers to herculase. Pfu refers to Pfu turbo polymerases.

the formation of primer dimers. As an alternative, we chose to introduce the *att*L sites using a two-step PCR amplification procedure. The first PCR reaction is performed using gene-/sequence-specific primers containing universal tails of 14 and 9 nucleotides, respectively, for the subsequent addition of the *att*L1 and *att*L2 sites. The nucleotide tails provide the homology for addition of the *att*L sites in the second round of PCR.

We further investigated whether truncated *att*L sites support efficient site-specific recombination. The primer pairs containing truncated *att*L sites were first evaluated for their ability to support PCR amplification using three different DNA polymerases (Figure 3). The PCR reactions carried out using *att*L1-T1/*att*L2-T1 (T1) and *att*L1-T2/*att*L2-T2 (T2) were successful with all DNA polymerases tested (Figure 3). In contrast, the PCR reactions using the *att*L1-T3/*att*L2-T3 (T3) and *att*L1-T4/*att*L2-T4 (T4) oligonucleotides worked with both HiFi Taq and Herculase (a high fidelity of blend DNA polymerase), but did not work with PFU. When the *att*L1 (SbfI) and *att*L2 (XhoI) primers were employed the PCR was only successful with HiFi Taq (Figure 3).

One-step LR recombination cloning

To assess our one-step cloning method, 10 different genes ranging in size from 756 to 3045 bp were amplified and the *att*L sites were introduced by PCR. Following gel purification, the amplified products containing the *att*L tails were used directly in the LR cloning reaction and electroporated into *E. coli*. The cloning efficiencies were determined by PCR-based screening of the resultant colonies. Specifically, insert confirmation was achieved by screening overnight cultures with either universal primers flanking the recombination sites or with gene-specific primers. Two different vectors carrying different regulatory elements and bacterial selectable markers were used to evaluate the LR cloning reaction. When the Spec-resistant vector was used, an average cloning efficiency of 86% was observed, with six of the genes having 100% (8/8) inserts (Table 2). A comparable cloning efficiency (81%) is observed when the Kan-resistant vector is used in the LR reaction (Table 2). We have successfully used the one-step LR reaction to clone hundreds of fragments ranging in size from 200 to 7500 bp (data not shown). While we find no clear correlation between the size of the insert and the cloning efficiency, the efficiency is dependant on the quality of the PCR product. Given that the *att*L regions are 95% identical, and primer-dimers are a significant source of false positives, it is essential to purify the PCR products prior to cloning. Failure to purify the products can result in a substantial increase in background due to cloning of primer-dimers.

Table 2. One-step recombination cloning using PCR products amplified with gene specific primer pairs tailed with *att*L1 and *att*L2 sequences

Vector	Insert (bp)	Colonies screened	Confirmed inserts
Kan	756	8	3
Kan	1209	8	8
Kan	1533	8	8
Kan	1542	8	3
Kan	1785	8	8
Kan	1953	8	8
Kan	1992	8	8
Kan	2016	8	6
Kan	3045	8	6
Spec	756	8	7
Spec	1209	8	8
Spec	1533	8	8
Spec	1542	8	2
Spec	1785	8	8
Spec	1953	8	8
Spec	1992	8	8
Spec	2016	8	6
Spec	3045	8	8
Mean cloning efficiency	84%		

The resultant products where cloned into two destination vectors harboring different selectable markers, Kan (kanomycin-resistant) or Spec (spectinomycin-resistant). Eight colonies from each cloning reaction were screened for the presence of the desired insert. In this pilot 113/144 (84%) of the clones screened contained the expected insert.

Next, we evaluated two different incubation times for the LR reaction, a 1 h incubation and 16 h incubation. Increasing the incubation time from 1 to 16 h results in a significant increase in the number of colonies (data not shown). Accordingly, our standard protocol for the LR reaction includes a 16 h. The longer incubation time is especially important when cloning fragments which are inefficiently amplified.

When the LR reactions were evaluated using four primer pairs T1, T2, T3 and T4, cloning efficiencies of 79, 83, 88 and 81% respectively were observed using the Spec-resistant destination vector (Table 3). Similarly, T1- and T2- amplified PCR products gave a 91 and 83% cloning efficiency using the Kan-resistant vector (Table 3). DNA sequencing analysis demonstrates that the expected recombination sites *att*B1 and *att*B2 are generated following the recombination reaction (Figure 4).

To evaluate the relationship between the concentration of PCR product and vector in the LR cloning reaction, serial dilutions of both the PCR product and the Kan-resistant vector were carried out. The results indicate that optimal cloning efficiencies are observed when the concentration of vector and PCR products are in the range of 100–300 ng in 50 µl reactions (data not shown). Linearization of the vector has no effect on the cloning efficiency (data not shown).

HTP cloning using a one step LR reaction

We have developed a one-step LR recombination method, which has been successfully employed for the HTP cloning of hundreds of genes and fragments amplified from a variety of sources. Based on our experience, >80% of the genes attempted were successfully cloned using our recombinational cloning method. The main factor for success is the ability to generate a discreet PCR product. Figure 5 depicts the PCR screening of 30 randomly selected cloning attempts. Using the Kan-resistant vector, 17/24 genes gave a 100% cloning efficiency, 5/25 gave 75% and 2/24 gave a 50% cloning efficiency (Figure 5). Among the six genes tested in this analysis using the Spec-resistant vector, five gave a 100% cloning efficiency and the sixth gave an 88% cloning efficiency (Figure 5). These results indicate that the one-step recombination cloning method is an efficient technique for directional cloning of large numbers of genes.

Table 3. One-step recombination cloning using PCR products amplified with gene specific primer pairs tailed with *att*L1 and *att*L2 sequences T1-T4

Primer pair	Vector	Insert (bp)	Colonies screened	Confirmed insert	Mean cloning efficiency (%)
attL1- attL2-T1	Spec	1785	8	5	79
		1953	8	7	
		2016	8	7	
attL1- attL2-T2	Spec	1785	8	8	83
		1953	8	7	
		2016	8	5	
attL1- attL2-T3	Spec	1785	8	7	88
		1953	8	7	
attL1-attL2-T4	Spec	1785	8	5	81
		1953	8	6	
attL1- attL2-T1	Kan	1785	8	8	91
		1953	8	8	
		2016	8	6	
attL1- attL2-T2	Kan	1785	8	8	83
		1953	8	7	
		2016	8	5	

The resultant products where cloned into the indicated destination vectors, Kan (kanomycin-resistant) or Spec (spectinomycin-resistant). Eight colonies from each cloning reaction were screened for the presence of the desired insert. Based on screening 24 colonies from each primer-vector combination mean cloning efficiencies ranging from 79% to 91% were obtained. For each of the individual cloning reactions, a minimum of five clones harboring the desired insert were obtained.

DISCUSSION

Over the course of a year we clone large numbers of genes and an efficient HTP cloning methodology is required to accommodate our cloning needs. Accordingly, methodologies such as recombinational cloning, which are not dependent on restriction enzymes and ligase have become increasingly popular. One example is the widely used Gateway® system (5) that provides good cloning efficiency, and readily facilitates transfer from one vector to another. This is a two-step procedure where the gene/fragment of interest is first cloned into an entry vector and subsequently transferred into one or several destination vectors (5). When the goal is to test the gene of interest in a single vector, a two-step procedure is unnecessary. Accordingly, we have developed a one-step method for recombinational cloning. Our cloning method provides a highly efficient alternative for HTP cloning, and eliminates the requirement to produce an entry vector.

The one-step recombination cloning is based on the well-characterized bacteriophage λ site-specific integration system. The cloning system is PCR based and requires two

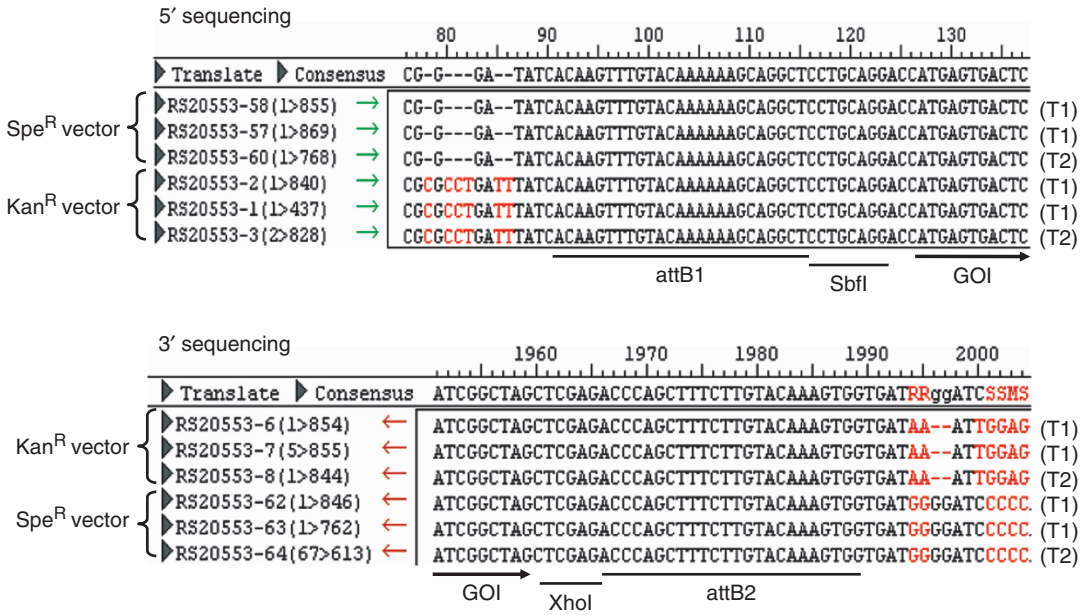

Figure 4. Sequence analysis of a gene cloned into Spec- and Kan-resistant vectors by onestep LR recombination cloning. T1 in parenthesis indicate the second PCR product was amplified from attL1-T1 and attL2-T1 oligonucleotides, and T2 indicates the second PCR product was amplified from attL1-T2 and attL2-T2 oligonucleotides.

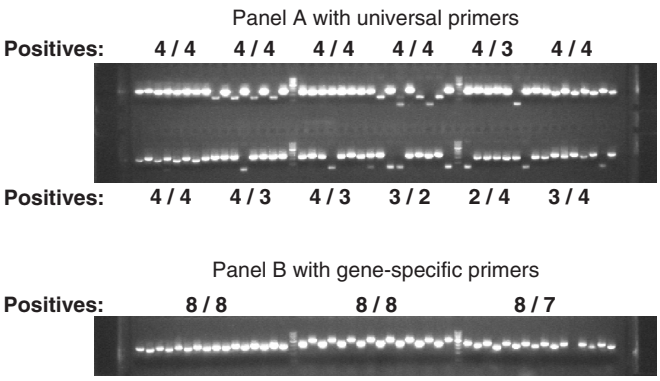

Figure 5. PCR confirmation of genes cloned into Kan-resistant vector (top panel) and Spec-resistant vector (bottom panel). Both cultured cells and plasmid DNAs were used for PCR confirmation. The 1 kb DNA marker (New England Biolabs) was used between every16 lanes. Samples were loaded using a multi-channel pipette, which loads every other well resulting in an interleaved loading pattern. In the upper panel, four individual colonies were screened for each clone using universal primers, which anneal to the vector sequences. Accordingly, clones without the insert amplify a small band of 200 bp. In the bottom panel, eight colonies from each clone were screened using gene-specific primers, so no band is observed for clones that do not contain the desired insert. While the use of gene-specific primers is useful for confirming the identity of a given clone, false negatives can arise due to PCR failures.

rounds of amplification. In the first round, the fragment/gene of interest is amplified with gene-specific primers containing short universal adaptors on their 5′ end. The adaptors provide the homology for the addition of the *att*L1 and *att*L2 sites in the second round of PCR. Following purification, the PCR product that is flanked by the *att*L or truncated *att*L-sites is cloned directly into the destination vector. The procedure is efficient and amenable to HTP cloning.

The requirement to tether the *att*-sites to the PCR primers prompted us to investigate the minimal size, which will support efficient integration. We identified sites ranging from 67 to 117 nt and 62 to 113 nt for *att*L1 and *att*L2, respectively, which support efficient recombination. The T1 oligonucleotide pair that is 67 and 62 nt, respectively for *att*L1 and *att*L2 are used for routine cloning applications. In this study, cloning efficiencies of roughly 90% were observed using oligos tethered with the T1 pair. This is in agreement with our historical success rate of >80% based on the cloning of hundreds of genes.

While all of the truncated *att*-sites supported efficient recombinational cloning (79–88%) for the Spec-resistant vector and (83–91%) for the Kan-resistant vector, the use of different *att*-sites of differing lengths does impact the PCR success rate. As summarized in Figure 3, we tested four truncated versions of *att*L1 (SbfI) and *att*L2 (XhoI) primer pairs T4 (79% of its original length), T3 (73%), T1 (57%) and T2 (45%). When the shortest primers T1 and T2 were used, the PCR reactions were successful with all three DNA polymerases tested (Figure 3). In contrast, the PCR reactions using the longer T3 and T4 primer pairs worked with both HiFi Taq and Herculase, but did not amplify using PFU. PCR products generated using any of the primer pairs all gave good recombination/cloning efficiencies ranging from 79% to 88% with the Spec-resistant vector. Similar results were obtained using the Kan-resistant vector (83–91% cloning efficiencies). Our results indicate that the shortest oligo pair tested (T2) is of sufficient length for recombinational cloning, but a somewhat better efficiency is observed using slightly larger oligonucleotides (T1). Using the T1 primer pair, a cloning efficiency of 91% was observed (Table 3). The T1 primer pair has been used for the recombinational cloning of hundreds of genes and a first pass success rate of >80% has been achieved. Sequence confirmation of the clones confirms that the expected sites *att*B1 and *att*B2 are faithfully generated in the recombination reaction.

We have developed a one-step recombination cloning method, which is not dependent on restriction enzymes, provides directional cloning, and the cloning efficiency is high. Our cloning uses truncated recombination sites to clone PCR products directly into destination/expression vectors, thereby bypassing the requirement for first producing an entry clone. The method is well suited to HTP applications, where the goal is to clone into a single expression vector.

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