

Methodology article

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The Flp double cross system a simple efficient procedure for cloning DNA fragments

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Abstract

Background: While conventional cloning methods using restriction enzymes and polynucleotide ligase are adequate for most DNAs, fragments made by the polymerase chain reaction are difficult to clone because the amplifying DNA polymerase tends to add untemplated nucleotides to the 3'-termini of the amplified strands. Conservative site-specific recombinases offer an efficient alternative to conventional cloning methods.

Results: In this paper I describe the use of the Flp site-specific recombinase for cloning PCR-amplified fragments. A DNA fragment is amplified with primers that contain at their ends inverted target sequences for Flp. Flp readily recombines these fragments *in vitro* into a vector that also contains two inverted Flp target sequences surrounding the α -complementing region of the *lacZ* gene of *E. coli*. The recombinants are conveniently detected as white colonies by the familiar blue/white screening test for *lacZ* activity. A useful feature of the system is that both orientations of the inserted DNA are usually obtained. If the recipient vector is cut between the two inverted Flp targets, Flp "heals" the double-strand break by inserting a linear fragment flanked by Flp targets.

Conclusion: This system ("The Flp Double Cross System") should be useful for cloning multiple PCR fragments into many sites in several vectors. It has certain advantages over other available recombinase-based cloning procedures.

Background

Cloning of DNA fragments is usually done by cleaving DNA and a vector with restriction enzymes, ligating the DNA molecules together and screening transformed bacteria for the desired recombinants [1]. The advent of the polymerase chain reaction (PCR) has facilitated the amplification of any piece of DNA whose sequence is at least partly known [2]. It is often desirable to clone directly a PCR fragment but because the amplifying DNA polymerase frequently adds extra residues to the 3' ter-

mini [3,4], one must commonly cut the fragment with a restriction enzyme or clone it into a special vector that contains protrusions that are complementary to the extended termini on the PCR fragment.

Conservative site-specific recombinases catalyze rearrangements of DNA at specific sequences [5-7]. They cleave and covalently attach to their target sites (for example, the Flp recognition target, FRT site, see Fig. 1a) and

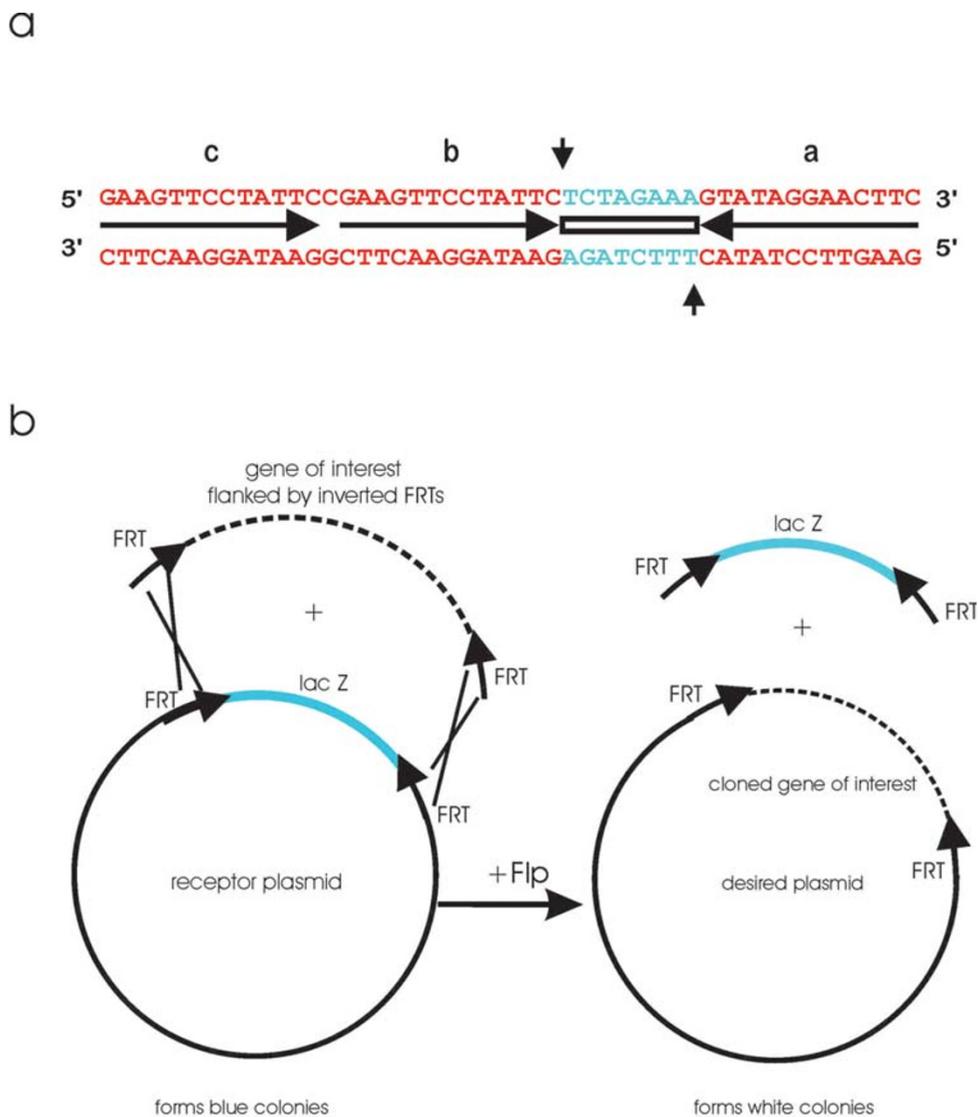


Figure 1

Figure 1

a). The structure of the FRT site. The FRT site consists of three 13 bp symmetry elements (horizontal arrows, a, b, and c, red letters) to which Flp binds in a site-specific manner. The 'a' and 'b' elements are in inverted orientation while the 'b' and 'c' elements are in direct orientation. The 'c' element is dispensable for recombination function but was included in all the substrates used here. The 'a' and 'b' elements are separated by an 8 bp asymmetric core region (open box, blue letters) across which recombination takes place. The vertical arrows indicate the two cleavage sites [24]. Flp makes a pair of staggered breaks at these sites, covalently attaching itself to the 3'-phosphoryl C residue at each site via the catalytic tryosine, residue no. 343[7].

b). General strategy for FRT double cross cloning. The receptor plasmid has the *lacZ* cassette flanked by inverted FRT sites. The gene of interest is PCR-amplified with primers that also contain inverted FRT sites at their ends. Flp promotes a double cross-over and replaces the *lacZ* cassette with the gene of interest. The receptor plasmid (left) causes its host bacterium to make a blue colony when plated on the indicator dye X-Gal. The desired plasmid (right) causes a white colony. Because the FRT sites are in inverse orientation, the gene of interest is not cut out. Rather Flp inverts its orientation so that both orientations are usually obtained.

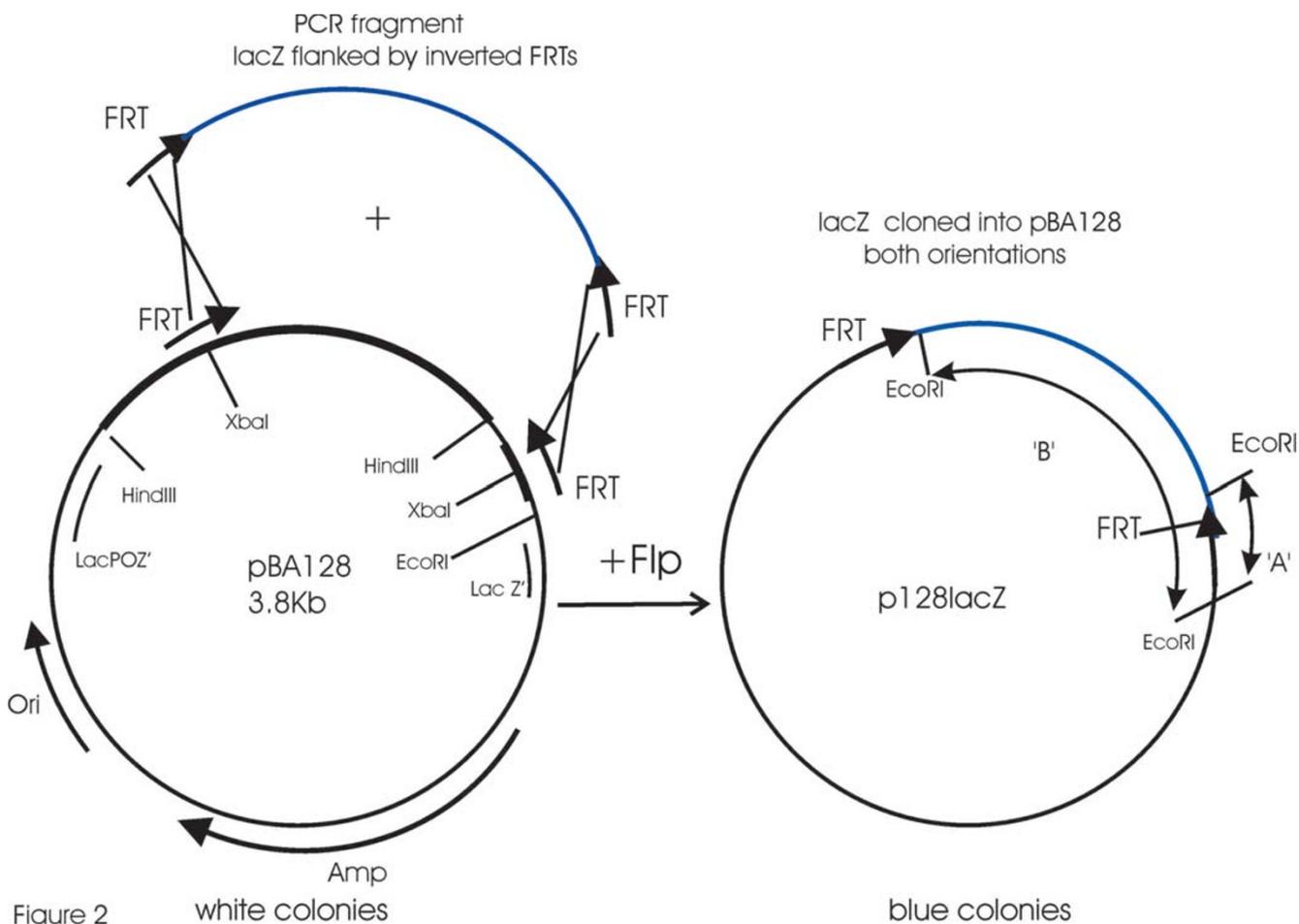


Figure 2
 a) Strategy for cloning the *lacZ* fragment into pBA128. The PCR-amplified *lacZ* fragment containing terminal inverted FRT sites is incubated with plasmid pBA128 and Flp. After transformation of XLI-Blue competent cells, the DNA from blue colonies is analyzed by *EcoRI* enzyme digestion. Colonies containing the parent pBA128 plasmid are white in color (left) whereas the p128*lacZ* transformants are blue (right).

they are able to recombine DNA with exquisite fidelity and very high efficiency.

Members of the integrase family of recombinases such as Cre of phage P1 and Flp of the yeast 2 μ M plasmid have been extensively used *in vivo* to engineer the genomes of several organisms [8,9]. Such tools have been powerful adjuncts to the study of development. Recently, members of the Integrase family have also been used for cloning DNA fragments *in vitro* [10,11]. While efficient, these systems require the prior construction of vectors and genetic selections to remove undesired products.

I describe here an *in vitro* system in which PCR fragments bearing two inverted targets of the Flp recombinase (FRT

sites) are recombined by the Flp protein into a vector that also contains two inverted FRT sites. Bacterial colonies containing the desired recombinants can be readily identified using the familiar blue/ white screening technique for the presence or absence of β -galactosidase [12,13].

Results

Rationale of the cloning system

I sought to develop a procedure that would facilitate the cloning of many PCR fragments into any vector. Commercially available systems [14] depend upon the availability of the particular vector from the supplier.

The present system (Fig. 1b) requires only that the user clones into the desired target site a receptor cassette that

Table 1: PCR primers used in this study

#	Primer name	Primer sequence (5'-3')	Features	Amplifies
1.	FRT 5' (Bam) <i>LacZ</i>	tcaggcgcgccgtcgaccatag gaagttcctatc tttctaga gaataggaacttc g gaataggaacttc GGATCC caaccgcctctcccgcgc	Buffer sequence FRT site <i>Bam</i> HI site <i>lac</i> sequence 97 nt	591 bp of <i>lac</i> promoter, operator and <i>lacZ</i> (including primers). 5' end begins at nt# 1081 of J01636 (G1146575)
2.	FRT inverse 3' (RI) <i>LacZ</i>	catggcgcgccgtcgaccatag gaagttcctatc tttctaga gaataggaacttc g gaataggaacttc GAATTC cggaaccaggcaagcg	Buffer sequence FRT site <i>Eco</i> RI site <i>lacZ</i> sequence 96 nt	591 bp of <i>lac</i> promoter, operator and <i>lacZ</i> (including primers). 5' end begins at nt# 1479 of J01636 (G1146575)
3.	FRT 5' (Bam) KvDMR	tcaggcgcgccgtcgaccatag gaagttcctatc tttctaga gaataggaacttc g gaataggaacttc GGATCC agtgggggcttcagaacatc	Buffer sequence FRT site <i>Bam</i> HI site KvDMR sequence 97 nt	1679 bp of intron 10 of human KCNQ1 gene(including primers). 5'end begins at nt #68364 of acces- sion # U90095
4.	FRT inverse 3' (RI) KvDMR	catggcgcgccgtcgaccatag gaagttcctatc tttctaga gaataggaacttc g gaataggaacttc GAATTC gattccagactccaattcca	Buffer sequence FRT site <i>Eco</i> RI site KvDMR sequence 97 nt	1679 bp of intron 10 of human KCNQ1 gene (including primers). 5'end begins at nt #66879 of acces- sion # U90095

consists of the *lac* operator/promoter region and the alpha-complementing N-terminus of the beta galactosidase gene surrounded by two inversely oriented FRT sites. Bacterial colonies containing the receptor vector stain blue in the presence of X-Gal dye [12,15]. One then amplifies the desired fragment with primers that contain terminal inversely oriented FRT sites. Flp catalyzes an efficient double crossover event between the PCR fragment and the receptor *lacZ* cassette causing replacement of the latter with the PCR fragment. The DNA is used to transform a suitable host and the colonies containing the recombinant clones are white when plated in the presence of X-Gal. A secondary benefit of this procedure derives from the fact that the FRT sites are in inverse orientation; once the fragment is inserted, Flp cannot cut it out again although it can readily invert it so that one will usually obtain the desired fragment cloned in both orientations [7]. This is in contrast to several cloning systems that rely upon the (reversible) formation of recombinase-catalyzed replicon fusions.

Cloning of the *lacZ* FRT cassette into plasmid pBA128

In order to create a test plasmid, I used PCR primers 1 and 2 (Table 1) to amplify a 398 bp sequence of the E. coli *lac* operon. In addition to the *lac* sequences, the primers contained two inversely oriented, 48 bp FRT sites as well as 23 nucleotides of 'buffer' sequence at their 5' termini. These buffer sequences contained three restriction enzyme recognition sequences (*Asc*I, *Sal*I and *Nde*I) to facilitate conventional cloning into other vectors although they were not used here. Primers 1 and 2 also contained a *Bam*HI and an *Eco*RI recognition site, respectively located 3' to the FRT sites to facilitate the determination of the orientation of the insert. This 592 bp fragment is called the "*lacZ*(FRT)² cassette".

To create a test receptor plasmid molecule I used Flp itself to recombine the *lacZ*(FRT)² cassette into plasmid

pBA128 (Fig. 2a). This pUC9-based plasmid contains two inversely oriented FRT sites 667 bp apart (Brenda J. Andrews, unpublished). I incubated pBA128 DNA (100–1000 ng, 15–150 nM of symmetry elements), the *lacZ*(FRT)² cassette (100 ng, 100 nM symmetry elements) and purified Flp protein (140 ng, 200 nM) together. The recombinant plasmids changed the color of the colonies containing them from white to blue. Flp catalyzed the appearance of blue colonies that was dependent upon the presence of the *lacZ*(FRT)² cassette. No blue colonies were obtained in the absence of the cassette or in the absence of Flp (data not shown). The frequency of blue colonies varied between 1 and 10%, depending on the relative concentrations of the vector and the fragment. One µg of vector yielded 42 blue colonies (2790 colonies total; 1.5%) whereas 100 ng of vector gave 55 blue out of 570 total colonies (9.6%). Restriction analysis showed that DNA isolated from blue colonies had the pattern expected of a Flp-mediated insertion of the *lacZ*(FRT)² cassette, creating plasmid "p128*lacZ*" (data not shown). Furthermore the *lacZ*(FRT)² cassette was inserted in both orientations with equal frequency. The plasmid DNA isolated from white colonies was the expected parental pBA128 DNA.

Although I used Flp to clone the *lacZ*(FRT)² cassette into the pBA128 vector, the cassette can also readily be cloned into any suitable restriction site in any vector by conventional techniques. After transformation of a suitable host, the desired blue colonies are easily identified.

Use of p128-*lacZ* plasmid for cloning of other PCR fragments

In order to show that the p128*lacZ* plasmid could serve as a receptor for other PCR fragments, I amplified a 1.5 Kb fragment of human DNA from the intron 10 of the *KCNQ1* gene on chromosome 11. Recombination of this fragment into p128*lacZ* was expected to create the two isoforms (See Fig. 3a). The primers also contained the same

FRT sites and buffer sequences as those on the *lacZ*(FRT)² cassette (primers 3 and 4, Table 1).

Analysis of the DNA from 8 white colonies obtained from the incubation with the KCNQ1 fragment showed that 6 of them contained an insert of the expected 1.6 Kb size when the DNA was cut with *Xba*I, an enzyme that cuts within the FRT sites (Fig. 3b, lanes 2, 3, 4, 6, 7, 9). The frequency of white colonies for this fragment was about 1%. Furthermore I confirmed by digestion with *Eco*RI that the experiment yielded approximately equal numbers of each orientation of the insertions (2 'A' isolates {Fig. 3c, lanes 3 and 6} and 4 'B' isolates {Fig. 3c, lanes 2, 4, 5, 7}). These experiments show that FLP can recombine a PCR fragment containing inverted FRT sites into a receptor vector containing an indicator *lacZ*(FRT)² cassette. A simple and familiar color screen yields a high frequency of recombinants in both orientations.

Use of linear receptor vector containing inverted FRT sites

It is well known that linearizing a circular DNA plasmid markedly reduces its efficiency of transformation of bacteria. It seemed possible that a linear plasmid containing two inverted FRT sites, one at each end, might be "healed" by a double crossover between the plasmid and a linear fragment catalyzed by FLP. The result would be a circular plasmid containing the insert identical to the fragment. I therefore linearized plasmid pBA128 by cutting it between the two inverted FRT sites with *Pst*I (Fig. 4a). The plasmid was then incubated in the presence of the *lacZ*(FRT)² cassette and FLP and the DNA was used to transform XL1-Blue competent *E. coli* cells. There was a large increase in the proportion of blue colonies that was dependent upon FLP and the presence of the *lacZ*(FRT)² cassette (Table 2). Linearization of the plasmid caused a 30-fold decrease in the transformation efficiency. FLP significantly reduced the transformation efficiency further, an effect that was mitigated by inclusion of the *lacZ*(FRT)² cassette in the reaction. This may have been due to the presence of exonuclease in the FLP preparation although the reactions were done in the absence of divalent metal. Furthermore, highly purified homogeneous FLP gave the same results. Importantly inclusion of the *lacZ* cassette in the reaction with FLP caused the appearance of greater than 50% of blue colonies. The appearance of blue colonies was dependent on inclusion of the *lacZ* cassette in the reaction (Table 2) and on the inclusion of FLP in the reaction (data not shown). The latter experiment excludes the possibility that the recombinants are being formed in *E. coli in vivo*.

To confirm that the transformants contained plasmids with the expected structure, I isolated DNA from 5 blue colonies and subjected it to digestion with *Xba*I and *Eco*RI. The results showed that all 5 of 5 blue colonies assayed

contained DNA that gave the expected patterns of digestion for insertion of the *lacZ*(FRT)² cassette (Figure 4a). As seen in Fig. 4b, all 5 isolates showed the expected 500 bp band upon digestion with *Xba*I (lanes 4–8). Furthermore the *lacZ*(FRT)² cassette was inserted in both orientations (Fig 4c, 'A' orientation lanes 4, 7; 'B' orientation, lanes 5, 6, 8). Similar results were obtained with two other linear plasmids that contained a cloned KCNQ1 cassette (data not shown).

Discussion

Advantages of FLP Double cross System

In this paper I describe a simple cloning procedure that uses FLP to recombine fragments of DNA that contain inverted FRT sites into a receptor vector that contains similarly oriented FRT targets. These FRT sites flank a cassette that imparts a color to transformant colonies, in this case the alpha-complementing fragment of *lacZ* that imparts a blue color. The system has several advantages over present cloning strategies, especially for the cloning of multiple PCR fragments into the same site of a vector. Firstly, because of the high specificity and simple requirements of FLP, the reaction is highly efficient. Since conservative site-specific recombinases like FLP attach covalently to their DNA substrate via a hydroxylated amino acid (tyrosine for members of the integrase family) no high-energy cofactor such as ATP is required. Furthermore FLP performs its reactions without the need for accessory factors such as those required by the lambda integrase (the host-coded IHF and the phage-coded Xis [6]).

Secondly, FLP catalyzes intramolecular excision or inversion reactions on DNA molecules containing two FRT sites as well as intermolecular reactions between molecules containing FRT sites [7]. The system described here uses the ability of FLP to catalyze two intermolecular reactions (or perhaps one intermolecular reaction followed by an intramolecular reaction) and thereby to insert a fragment into the receptor plasmid. Once the double crossover has occurred, FLP cannot excise the insert from the plasmid because the FRT sites are in inverted orientation. A useful feature of this system is that FLP efficiently will invert the insert between the FRT sites with the result that one almost always obtains both orientations of the insertion. This can be very helpful in examining the orientation-dependence of a *cis*-acting sequence or in generating sense and antisense RNA probes.

Thirdly, the FLP double cross system is simple in concept and in execution. It uses the familiar blue-white screening technique to identify the desired recombinant clones. It is therefore adaptable to the cloning of any fragments into any site in any vector. The prerequisites are the construction of a single vector that contains the PCR-amplified

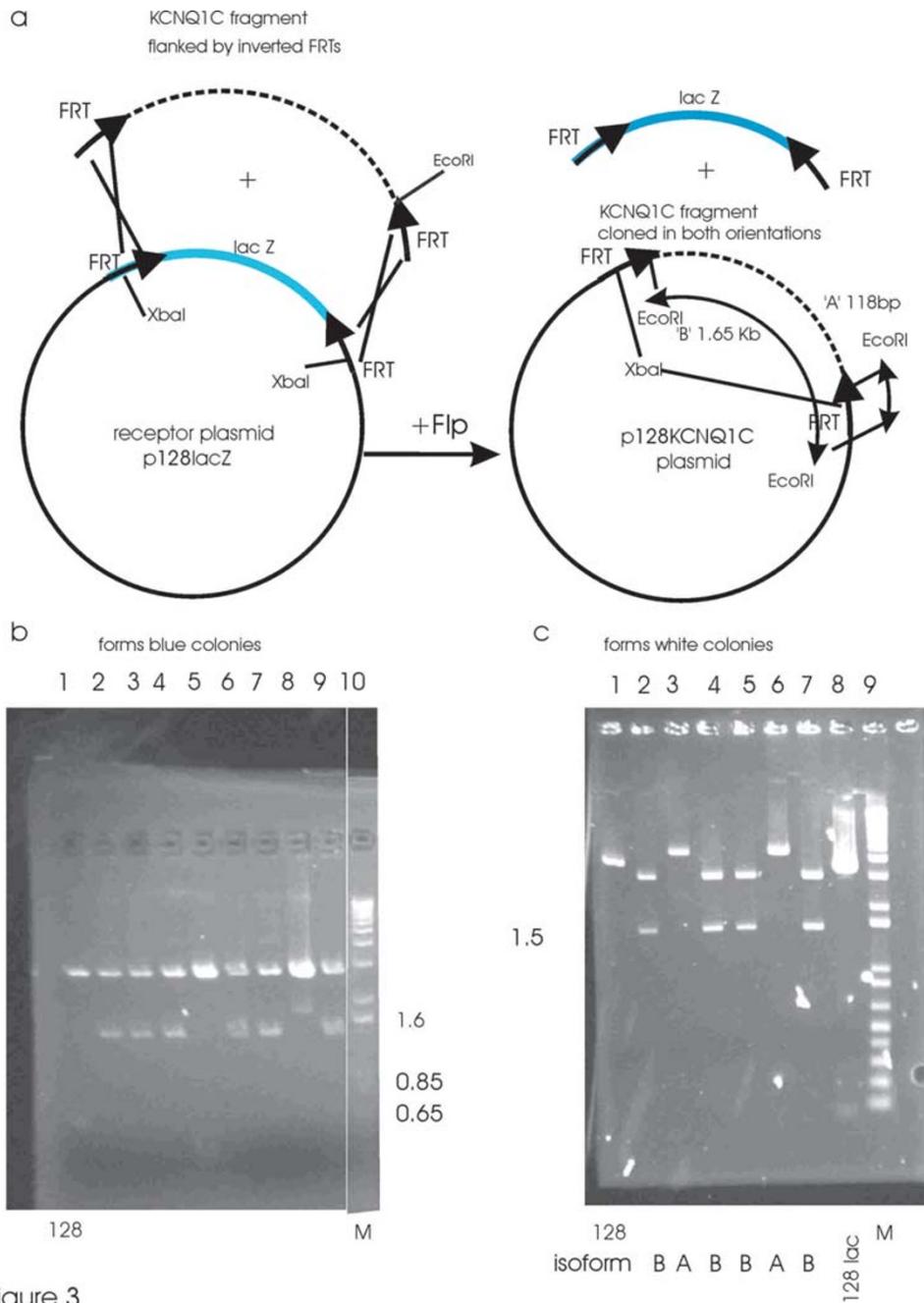


Figure 3

Figure 3

a) Cloning of a 1.65 Kb fragment of human DNA into receptor plasmid p128lacZ. Schematic diagram. A 1.65 Kb fragment from intron 10 of the human KCNQ1 gene (dashed arc) was amplified using the primers 3 and 4 and subjected to Flp double cross cloning into receptor plasmid p128lacZ. The resultant recombinants transform XLI-Blue cells to form white colonies (right). The KCNQ1 fragment replaces the lacZ cassette in either orientation. In the 'A' orientation EcoRI digestion yields a 118 bp fragment while in the 'B' orientation a 1.65 Kb fragment is seen. b) Agarose gel analysis of DNA isolated from white colonies after Flp double cross cloning of KCNQ1 fragment into 128lacZ receptor plasmid. XbaI. Six of the plasmids from 8 white colonies (lanes 2–4, 6, 7 and 9) show the expected 1.6 Kb fragment. Lane 1-plasmid pBA128; lane 10-marker DNAs (M, 1 Kb+marker DNA). c) The DNAs shown in b) were then digested with EcoRI (lanes 1–9). Lanes 2–7 show the same DNAs as in panel b) from lanes 2, 3, 4, 6, 7, 9. Four of them show a 1.5 Kb fragment diagnostic of the B form of the plasmid (lanes 2, 4, 5, 7,) whereas the DNA from two of the six is in the A form (lanes 3 and 6,). The 118 bp fragment cannot be seen on this gel. Lane 9-1Kb+ marker DNAs.

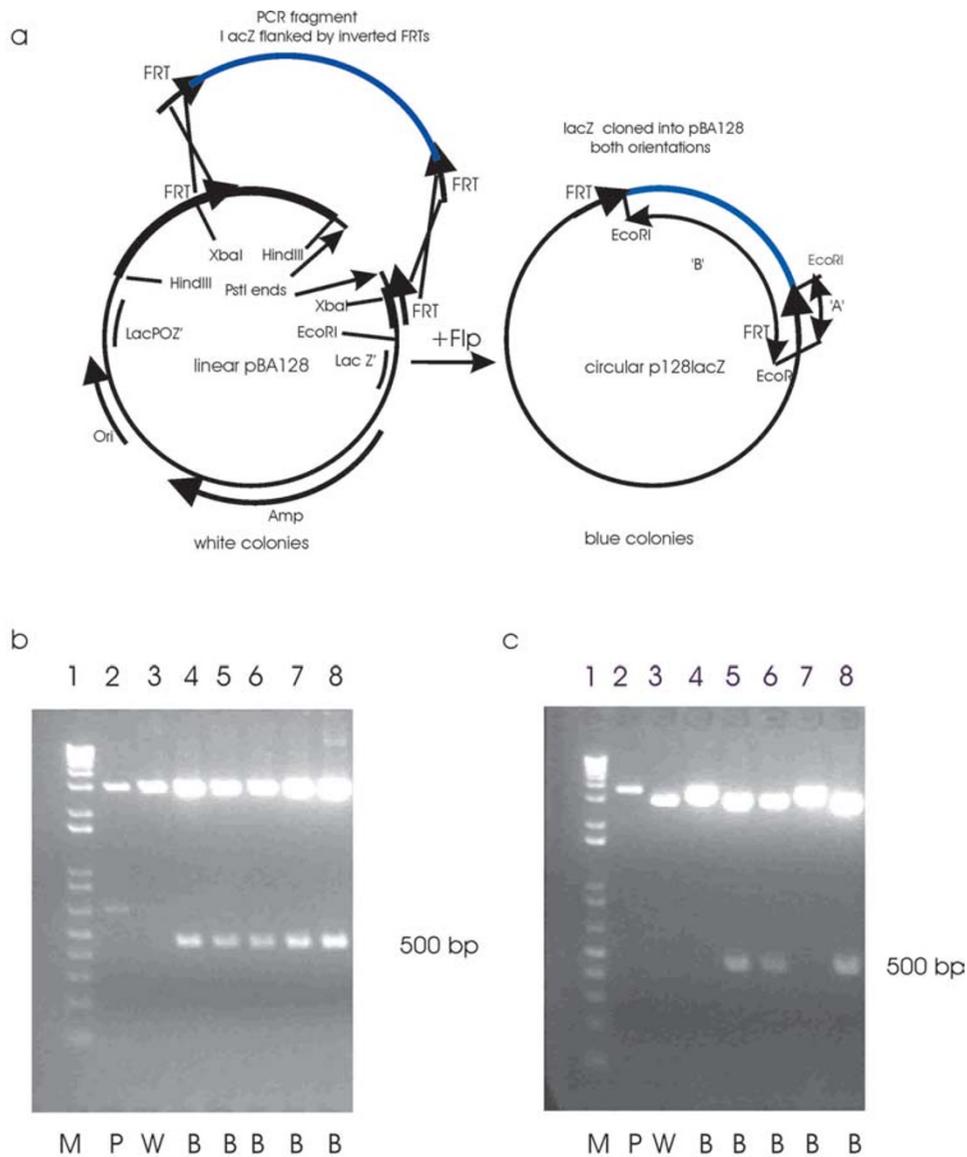


Figure 4

Figure 4

Flp double cross cloning into a linear vector. a) Cloning of the *lacZ* cassette into linear pBA128 plasmid. The plasmid is linearized with *Pst*I and incubated in the presence of the *lacZ* cassette and Flp (left). Flp catalyzes the insertion of the *lacZ* cassette into the linear vector forming a circular p128*lacZ* derivative (right). The insertion takes place in either orientation with the formation of the A and B isoforms. Colonies harboring the desired plasmids are blue when plated in the presence of X-Gal. The two isoforms can be distinguished by digestion with *Eco*RI. b) Agarose gel electrophoretic analysis of DNA from Flp double cross cloning of *lacZ* cassette into linear vectors. The *lacZ* cassette was incubated with linear pBA128 DNA (lanes 2–8). The DNA resulting from transformation of competent XLI-Blue cells was digested with *Xba*I. The DNAs in corresponding lanes shown in panels b) and c) were isolated from the same colony. M-1Kb+ marker DNAs. Blue colonies (B) all showed a 512 bp band diagnostic of insertion of the *lacZ* cassette (lanes 4–8) whereas the white colony (W) did not (lane 3). The parent plasmid (P, pBA128, lane2) showed band of 672 bp. c) *Eco*RI digestion. The isolates in lanes 5, 6, 8, showed the 512 bp band indicative of the 'B' orientation whereas those in lanes 4, and, 7 showed no such band since the 118 bp, indicative of the 'A' orientation band was not visible in this gel. Note however that the top vector band is migrating more slowly than those DNAs having the 'B' orientation. P-parental DNA was pBA128 (lane2) digested with *Eco*RI.

Table 2: Flp cloning using linear plasmid DNA*

Addition/reaction	1	2	3	4
Circular pBA128	+			
Linear pBA128	-	+	+	+
Lac Z cassette	-	-	-	+
Flp	-	-	+	+
Colonies/ μ g	6×10^7	1.8×10^6	1.5×10^4	3×10^5
% blue colonies	0	0	0	56%

*The reactions (10 μ l) contained 100 ng of the indicated plasmid DNA, and where indicated, 100 ng of the purified *lacZ* cassette and 130 ng of S300 fraction of Flp. The reactions were processed as described in the Methods and 1 μ l aliquots were used to transform competent XL1-blue *E. coli* cells (Stratagene). Transformed cells were plated on LB agar plates containing ampicillin, IPTG and X-Gal as described in the Methods.

lacZ(FRT)² cassette by conventional cloning and a source of Flp. The receptor vector can be used to clone many fragments that have been amplified using primers containing terminal FRT sites. Although Flp is not available commercially and is not produced abundantly in *E. coli* when histidine-tagged (A. Shaikh and P.D. Sadowski, unpublished), nevertheless, ample amounts for the cloning method described here can be made in a 2-column procedure [16]. A two-column procedure for purifying His-tagged mutant Flp protein was recently described [17].

Comparison with Other Recombinase-based Cloning Systems

The Flp double cross system has advantages over currently available cloning systems that use site-specific recombinases or topoisomerase. The Gateway™ cloning system of Invitrogen [10] uses the lambda integrase reaction to transfer a gene of interest flanked by *attL* sites from an "entry clone" into a "destination vector". While elegant in its design, this system may be conceptually difficult for the non-expert to understand. Furthermore it requires the prior construction of the entry clone by the user and depends on the availability of the appropriate destination vector from the vendor. The Flp system is simple to understand and to execute and requires only the construction of a single receptor vector by the user.

Invitrogen and Clontech also sell a Cre-based cloning system (The Echo™ Cloning System and the Creator™ Gene Cloning and Expression System, respectively). Both require the construction of donor vectors, the purchase of acceptor vectors and genetic selections because of the ready reversibility of the Cre reactions. The Flp double cross system circumvents the reversibility problem by using two inverted FRT sites. Because of the high efficiency, a simple color screen suffices to detect the desired recombinants. Invitrogen also markets the TOPO® Cloning System [14] that uses a vector containing topoisomerase that is covalently attached to ligate the PCR fragment

to the vector. While highly efficient, it depends on the availability of the appropriate topoisomerase-containing vectors from the vendor.

Improvements to the System

In the present experiments I used primers that contained the full 48 bp sequence of the FRT site flanked by 23 nucleotides of 'buffer' sequence. Thus the PCR primers are rather long and hence expensive. It is likely that the primers could be substantially shortened by deleting the buffer sequences and/or the 'c' symmetry element. It is well known that the third 13 bp symmetry element ('c', See Fig. 1a) is not needed for efficient recombination by Flp *in vivo* and *in vitro* [18,19]. The FRT site contains a near-perfect 13 bp palindrome, (the a and b symmetry elements, Figure 1a) which may cause unpredictable effects when the vector is used. Control experiments are needed to exclude such effects that may be attributable to the FRT sites. A detailed study of the importance of each position in the symmetry elements to the Flp recombination reaction has been carried out [20]. It should be possible using these studies as well as the recent co-crystal structure of Flp and the FRT site [21] to design FRT sites that would be functional and yet not interfere with translation or other processes. Likewise it should be possible to design a functional FRT site that is not palindromic. A selection system for Flp variants with altered FRT site specificity has recently been described [22]. Directional insertions could also be achieved using directly oriented FRT sites that have non-homologous spacer regions.

Although I chose the *lacZ* α -complementation system as the indicator because of its familiarity and convenience, other indicator systems are possible. For example, I amplified a PCR fragment containing the green fluorescent protein [23] driven by the *lac* promoter and flanked by inverted FRT sites. Flp recombined this fragment into the pBA128 vector. The recombinants were easily detected as green fluorescent colonies under long-wavelength ultraviolet light. However the green fluorescent protein

expressed from the *lac* promoter seemed to be toxic to the *E. coli* cells. Further experiments are being done with this system. In developing the test system I made extensive use of the *lacZ*(FRT)² cassette that functioned rather efficiently. It is my experience that different PCR fragments recombined with different efficiencies. For example the KCNQ1 fragment, which is a part of CpG island from human DNA did not recombine as efficiently as the *lacZ*(FRT)² cassette. The reasons for the variability in cloning efficiencies are being investigated.

Conclusion

The present paper describes a simple and efficient cloning strategy using the Flp recombinase. The method has certain advantages over currently available commercial procedures and may be extended to other recombinases; it could be applied *in vivo*. The vectors and Flp protein used in this paper are available from the author.

Methods

Media and growth conditions

Bacteria were grown in LB medium supplemented with 100 µg per ml of ampicillin according to standard methods [1]. Competent *E. coli* XL1-Blue cells (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^q ZΔM15 Tn10* (Tet^r)]) were purchased from Stratagene (La Jolla, CA, USA, cat. # 200249). Transformation and staining colonies for beta galactosidase with X-Gal were done according to the manufacturer's instructions.

DNA isolation

Plasmid pBA128 is a derivative of plasmid pBA 112 [24] into which a 912 bp *Hind* III fragment of the 2 µM plasmid (nt #s105-1017) was inserted into the *Hind*III site. The two FRT sites are in inverted orientation with respect to one another. The plasmid DNA was isolated as described previously [25]. Small-scale preparations of plasmid DNAs were done using the QIAprep Spin Miniprep Kit (QIAGEN Inc, Valencia, CA, USA) or the alkaline-iodine lysis procedure [26]. PCR fragments were purified from agarose gels using the QIAGEN QIAquick Gel Extraction Kit. PAC DJ74K15 DNA (Accession no.90095) was isolated from *E. coli* DH10B (Δ (*mrr-hsd RMS-mcrBC*)*mcrA recA1*) containing PAC DJ74K15 DNA by the alkaline lysis method followed by phenol extraction and ethanol precipitation [27]. *E. coli* DNA was isolated from strain N99 (*galk2, strep^r*) [28] and was the gift of Dr. Barbara Funnell.

Enzymes

Flp (70% pure) was purified as described [16] up to the Sephacryl S300 step. Restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA) and were used according to the manufacturer's instructions. *Taq* DNA polymerase, PCRx enhancer and PCRx enhancer buffer solution, MgSO₄, deoxynucleoside triphosphates

and oligodeoxynucleotide primers (see table 1) were purchased from GibcoBRL(now Invitrogen, Carlsbad, CA, USA). The primers were desalted and used without further purification.

PCR amplification conditions

i) *LacZ*. The reaction mixtures (50 µl) contained 1xPCRx Enhancer buffer, 3 mM MgSO₄, 0.25 mM of each of the four deoxynucleoside triphosphates, 4 µM of each of primers 1 and 2, see Table 1), 100 ng of *E. coli* DNA and 2.5 units of *Taq* DNA polymerase. The cycling conditions were: 95°, 2 min, {95°, 45 sec, 55°, 50 sec, 72°, 2 min} repeated 30 times, 72°, 10 min, cool to 4°.

ii) KCNQ1. The reaction mixtures (50 µl) were the same as above except contained 4 µM of each of primers 3 and 4, (Table 1) and 20 ng of PAC DJ74K15 DNA. The cycling conditions were: 95°, 2 min, {95°, 45 sec, 60°, 45 sec, 72°, 2 min} repeated 30 times, 72°, 10 min, cool to 4°.

Agarose gel electrophoresis

Agarose gels (1.5%, w/v) were run in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0 [13] and stained with ethidium bromide.

Flp double cross reactions

The reaction mixtures (10 µl) contained 50 mM Tris-Cl buffer, pH 7.4, 33 mM NaCl, 1 mM EDTA, 100 ng (~0.15 pmol {15 nM} of symmetry elements), or 1000 ng (1.5 pmol {150 nM} symmetry elements) of plasmid substrate and ~100 ng of PCR fragment (1.0 pmol {100 nM} of symmetry elements for *LacZ* cassette; 0.375 pmol {37.5 nM} of symmetry elements for the KCNQ1 fragment) and 140 ng of S300 Flp preparation, (2 pmol of protein, 200 nM). Reactions were incubated for 30 min at 30°, heated at 65° for 5 min and then chilled on ice. One µl aliquots were used to transform 2-mercaptoethanol-treated competent XL1-Blue cells (Stratagene).

Abbreviations

Bp-base pair(s); IPTG-isopropylthio-β-D-galactoside; nt (s)-nucleotide(s); PCR-polymerase chain reaction; X-Gal-5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside.

Author's contributions

I performed all the experiments described here. Flp protein was made by Donna Clary. See also Acknowledgements below.

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