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A Rapid, Efficient and Economical Method for Generating Leishmanial Gene Targeting Constructs

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Abstract

Targeted gene replacement is a powerful tool in *Leishmania* genetics that can be time-consuming to implement. One tedious aspect that delays progress is the multi-step construction of gene targeting vectors. To accelerate this process, we developed a streamlined method that allows the assembly of a complete targeting vector from all its constituent parts in a single-step multi-fragment ligation. The individual components to be assembled are flanked by sites for the restriction endonuclease SfiI that generates nonidentical, non-palindromic three base 3'-overhangs designed to allow annealing and ligation of the parts only in the proper order. The method was optimized by generating constructs for targeting the *Leishmania donovani* inosine monophosphate dehydrogenase gene (*LdIMPDH*) encoding six different drug resistance markers, and was found to be rapid and efficient. These constructs were successfully employed to generate heterozygous *LdIMPDH* gene replacement mutants. This method is adaptable for generating targeting vectors for a variety of species.

Keywords

Leishmania; gene targeting; gene knockout; homologous recombination; multi-fragment ligation; SfiI restriction endonuclease

Targeted gene replacement via homologous recombination has been an invaluable tool for the genetic dissection of important metabolic and virulence pathways in *Leishmania* species [1], as well as for many other protozoan parasites [2]. The general experimental approach for the genetic manipulation of model organisms is essentially the same: DNA sequences of sufficient length to direct homologous recombination flanking the gene to be targeted - referred to herein as 5'- and 3'-targeting sequences (TS) - are independently isolated and joined to an alternative gene (i.e., drug resistance gene) that allows selection of cells in which the appropriate integration event has occurred. The most common method for generating leishmanial and other parasite gene targeting constructs involves the sequential cloning of 5'-TS and 3'-TS DNAs into a vector encoding a drug resistance cassette flanked by restriction sites [3]. This multi-step process can be time consuming and is complicated by the fact that commonly used vectors have limited restriction sites for TS insertion, exchange of drug resistance markers, and excision of the targeting cassette from the vector backbone

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prior to transfection. The ability to assemble the complete gene targeting construct in a single step would greatly enhance the throughput of gene replacement studies. Numerous techniques have been described for the simultaneous assembly of multiple DNA fragments, including variations on ligation-independent cloning [4–6], overlap extension polymerase chain reaction (PCR) [7], site-specific recombination [8], and recombination in *Escherichia coli*, i.e., "recombineering"[9]. Some of these methods have been used to construct gene targeting constructs for various trypanosomatids [10,11]. However, these techniques can involve tedious, complicated, or unfamiliar technology [5,6,8,9,12], require expensive kits or reagents [4,8], and are complicated by inefficiency [13].

To overcome these many impediments to gene replacement in *Leishmania*, we have developed a simple, cost-effective, and efficient method (see Fig. 1) for construction of leishmanial targeting constructs that is based on the properties of the SfiI restriction endonuclease and requires only readily available and familiar technologies, (i.e., PCR, restriction digestion, and DNA ligation). The SfiI restriction endonuclease was chosen as the basis of this multi-fragment ligation strategy for several reasons. First, the recognition sequence for SfiI, GGCCN**NNN**NGGCC, is an interrupted palindrome that generates an asymmetric three base 3'-overhang (underlined and in boldface type in the sequence) upon DNA cleavage that can only ligate to complementary overhangs but not to itself nor to overhangs from non-identical SfiI sites. SfiI sites designed to produce non-identical 3'-overhangs incorporated at the ends of the individual targeting vector components allows the directional and ordered assembly of the targeting vector in a single ligation reaction (see Fig. 1C). Second, the eight base pair recognition sequence of SfiI should only rarely occur within any given TS, rendering this strategy applicable to the generation of targeting constructs for virtually any leishmanial gene. Indeed, examination of one megabase of *L. infantum* genome sequence identified SfiI sites with a frequency of approximately one in every 38 kb. If a TS encompasses an SfiI site, alternative restriction enzymes with six base pair recognition sequences that are also interrupted palindromes are potentially available for generating compatible 3'-overhangs (see Supplementary Protocol). Third, SfiI has been utilized by other groups for the ordered assembly of multiple DNA fragments and the method has proven to be quite efficient [13,14]. While the use of SfiI in other strategies for producing gene targeting constructs has been described [12,15], none of these schemes involve the simultaneous assembly of a complete targeting vector by single-step ligation of all of the constituent components.

Standard targeting vectors are normally composed of four parts: a minimal plasmid backbone to permit selection and propagation in *Escherichia coli*, a drug resistance cassette to allow selection in *Leishmania*, and a 5'-TS and a 3'-TS to facilitate gene replacement via homologous recombination. In the multi-fragment ligation method, all four parts are digested with SfiI, gel purified, and combined in a single ligation reaction to generate the complete targeting construct. As depicted in Fig. 1A, the minimal plasmid backbone is donated by the pBB plasmid that contains two incompatible SfiI sites (SfiI-A and SfiI-D) flanking a stuffer fragment that allows the plasmid backbone fragment to be readily distinguished from uncut vector during gel purification. An expression cassette encoding one of six possible drug resistance genes currently available for *Leishmania* transfection [1,16–18] is donated by the corresponding pCR-DRC plasmid (Fig. 1A) and is also flanked by incompatible SfiI sites (SfiI-B and SfiI-C). Importantly, the SfiI overhangs encoded by the plasmid backbone fragment and the drug resistance cassettes cannot ligate to each other, but, rather, require the complementary SfiI overhangs provided by the 5'- and 3'-TS PCR fragments (Fig. 1B) to serve as a bridge between them (Fig. 1C). The complete targeting vector (Fig. 1D) encodes ampicillin resistance and the fact that the pCR-DRC plasmids are kanamycin-resistant eliminates the possible contribution of uncut pCR-DRC plasmid to the occurrence of background colonies following transformation of the ligation reaction. All the

drug resistance cassettes are flanked by restriction sites for the rare cutting enzymes SbfI and AscI to facilitate the ready exchange of drug resistance cassettes between targeting constructs (i.e. pTRG in Fig. 1D) and the pCR-DRC plasmids (Fig. 1A). The targeting cassette can be conveniently excised from the plasmid backbone using either PacI or PmeI endonucleases, whose eight bp recognition sequences occur rarely.

To demonstrate the efficacy of the multi-fragment ligation method for targeting vector construction, we generated constructs for targeted replacement of the *L. donovani* inosine monophosphate dehydrogenase (*LdIMPDH*) gene [19]. As a first step, the conditions for the four-way ligations were optimized by varying the molar ratio of inserts (3'- and 5'-IMPDH TS fragments and puromycin (PAC) or phleomycin (PHLEO) drug resistance cassettes) to plasmid backbone fragment in trial ligation reactions. While the transformation efficiencies were uniformly high for all insert to vector ratios tested, the percentage of clones containing plasmids with the correct structure was consistently lower at the 0.5:1 molar ratio (Table 1). SfiI digestion of plasmid DNA from twenty colonies each from the 2:1 ratio ligations of both the PAC and PHLEO *LdIMPDH* targeting constructs revealed that 85% and 75%, respectively, had the correct structure (data not shown) confirming the efficiency of the method at this ratio. Therefore, a 2:1 insert to vector ratio was employed for construction of *LdIMPDH* targeting constructs encoding neomycin (NEO), hygromycin (HYG), blasticidin (BSD), and nourseothricin (SAT) expression cassettes, since it consistently allowed smaller ligation volumes to be used and yielded a large number of colonies containing a high percentage of targeting vectors with the expected structure in each case (data not shown).

While the overall number of colonies and the percentage of colonies containing the correct construct was high, it was noted that all of the incorrect plasmids could be readily identified as contaminating pBB-GFP plasmid (data not shown). Other systems for simultaneous assembly of multiple DNA fragments reduce the occurrence of background by including a conditionally lethal gene such as *ccd*B or *sac*B in donor vectors [8,12]. To reduce the potential for background colonies derived from the plasmid backbone donor plasmid, the GFP stuffer fragment of pBB-GFP was replaced with a chloramphenicol resistance/ccdB expression cassette to generate plasmid pBB-Cm^R-ccdB. This plasmid cannot be propagated in standard *E. coli* strains used for cloning but instead requires a specialized bacterial strain expressing the antitoxin to CcdB encoded by the *ccd*A gene [20]. When pBB-Cm^R-ccdB was used as the plasmid backbone donor in a four-way ligation to generate *LdIMPDH* targeting constructs containing a PAC cassette, no background was observed (data not shown). In fact, in eight additional targeting constructs have been produced in our laboratory using pBB-Cm^R-ccdB as the plasmid backbone donor and no background has been observed (A. Fulwiler and R. Soysa, unpublished observations).

To establish the utility of constructs generated by multi-fragment ligation for targeted gene replacement, *LdIMPDH* targeting constructs encoding BSD-, HYG-, NEO-, PHLEO-, and PURO resistance cassettes were transfected into wild type *L. donovani* parasites and plated on the appropriate selective medium. Southern blot analysis indicated that each targeting construct was capable of generating *IMPDH*/*impdh* heterozygotes with the expected genomic structure (Fig. 2).

The multi-fragment ligation strategy presented here for production of leishmanial gene targeting vectors is markedly faster than traditional approaches [3] and is significantly cheaper and more straightforward than alternatives [11]. Using this technique, we routinely generate gene targeting constructs in three to four days from the time of TS fragment PCR to confirmation of vector structure by restriction analysis. The general strategy is adaptable to the generation of targeting constructs for other parasites and genetically manipulable organisms by simply producing species-specific selectable markers flanked by the

appropriate SfiI sites. All of the components of this system are available by request from the authors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AMP^R	ampicillin resistance gene
BSD	blasticidin resistance
Cm^R	chloramphenicol resistance gene
GFP	green fluorescent protein
HYG	hygromycin resistance
KAN^R	kanamycin resistance gene
LdIMPDH	*Leishmania donovani* inosine monophosphate dehydrogenase gene
NEO	neomycin resistance
PAC	puromycin resistance
PHLEO	phleomycin resistance
SAT	nourseothricin resistance
TS	targeting sequence

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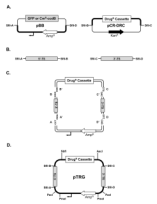

Fig. 1.

Strategy for assembly of gene targeting vectors via multi-fragment ligation. This method is modular in design with each component derived independently prior to simultaneous assembly in a single ligation reaction. (A) Plasmid pBB serves as the donor of a minimal (2165 bp) plasmid backbone (denoted by the thick black line) that encodes ampicillin resistance (AMP^R- open arrow) and a plasmid replication origin (small black arrow). There are two versions of pBB, pBB-GFP (Genbank accession no. **HQ416901**) and pBB-Cm^R-ccdB (Genbank accession no. **HQ416902**), that encode either GFP (~800 bp) or Cm^R-ccdB (~1600 bp) stuffer fragments, respectively, flanked by non-identical SfiI restriction sites (SfiI-A and SfiI-D). Drug resistance cassettes (thick black line) are donated by the pCR-DRC plasmids that were constructed by cloning drug resistance cassettes (~3 kb) PCR amplified with primers encoding unique SfiI restriction sites (SfiI-B and SfiI-C) into pCR®-Blunt II-TOPO®. Six pCR-DRC plasmids were constructed that contain drug resistance cassettes conferring resistance to either neomycin/G418 (pCR-NEO), hygromycin (pCR-HYG), phleomycin (pCR-PHLEO), puromycin (pCR-PAC), blasticidin (pCR-BSD), or nourseothricin (pCR-SAT). A detailed description of pBB and pCR-DRC plasmid construction is provided in the supplementary materials and methods. For all of the drug resistance cassettes, the appropriate trans-splicing and polyadenylylation of the drug resistance gene is directed by 5'- and 3'-flanking sequences, respectively, derived from the *L. major DHFR-TS* gene as described [1] (B) The 5'- and 3'-targeting sequences (TS) are generated by PCR amplification using primers that add SfiI restriction sites SfiI-A and SfiI-B (5'-TS) or SfiI-C and SfiI-D (3'-TS) (see Supplementary Protocol). (C) Targeting vector assembly. The 5'- and 3'-TS PCR fragments, along with the pBB and pCR-DRC donor plasmids are digested with SfiI and the appropriate DNA fragments are agarose gel purified and combined in a ligation reaction. Each DNA fragment has unique three base 3'-overhangs on the sense (indicated by A, B, C, D) and antisense (indicated by A', B', C', D') strands that can only anneal to complementary overhangs. (D) The assembled targeting vector (pTRG) contains restriction sites for exchange of drug resistance cassettes (SbfI and AscI) and release of the targeting fragment from the plasmid backbone (PacI or PmeI). Note that the plasmids are not drawn to scale.

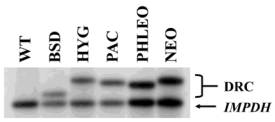

Fig. 2.

Southern blot analysis of Δ*impdh/IMPDH* parasites. Total genomic DNA (~2 μg) from wild type *L. donovani* (lane 1), or strains in which one *IMPDH* allele has been replaced by BSD (lane 2), HYG (lane 3), PAC (lane 4), PHLEO (lane 5), or NEO (lane 6) cassettes was digested with XhoI, fractionated on a 1% agarose gel, and blotted onto a nylon membrane. The blot was hybridized under high stringency conditions with a probe to the 3'-*IMPDH* TS. Bands above the *IMPDH* band correspond to the targeted *IMPDH* allele and vary in size in accordance with the sizes of the drug resistance cassettes (labeled DRC). Targeted replacement of *LdIMPDH* using a targeting construct containing a SAT cassette (pTRG-*IMPDH*-SAT) was not attempted.

Table 1

Optimization of multi-fragment ligation conditions

	Inserts : Plasmid Backbone Ratio	Number of Colonies	Transformation efficiency (cfu/μg)	Positive clones (%) (N=4)
PHLEO	0.5:1	450	3.2×10^6	25
	1:1	900	6.4×10^6	75
	2:1	1180	8.4×10^6	75
	3:1	1500	1.1×10^7	75
PAC	0.5:1	524	3.7×10^6	25
	1:1	1110	7.9×10^6	75
	2:1	1467	1.0×10^7	100
	3:1	1880	1.3×10^7	75
pUC19	-	2000	4.8×10^8	-
Plasmid backbone fragment	-	70	5.0×10^5	-

The molar ratio of inserts (5'- and 3'- *IMPDH* TS fragments and the drug resistance cassette) to plasmid backbone was varied with respect to 10 ng of the plasmid backbone. The Inserts: Plasmid Backbone Ratio denotes the molar ratio of each insert fragment relative to the plasmid backbone. The number of colonies from plating 100 μl (21% of total transformation volume) of *E.coli* transformed with 2 μl ligation reaction (representing 0.67 ng plasmid backbone fragment) is presented. Transformation efficiencies are given as colony forming units per μg of DNA and are calculated based on 0.67 ng of plasmid backbone fragment per transformation. Plasmid pUC19 (0.02 ng) was included to assess the transformation efficiency of the lot of commercially prepared competent *E. coli* used in this experiment. Transformation of a ligation reaction performed using only 10 ng of the purified plasmid backbone fragment (originating from the same batch used in inserts:vector ratio optimization) served to indicate the contribution of pBB-GFP to background. Further assessment of transformants derived from ligation reactions using 2:1 inserts to plasmid backbone ratio showed that the percentage of clones positive for the correct PHLEO- or PAC-containing targeting vector was 75% and 85%, respectively (N=20).