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Technical Report

Improved Production and Purification of Minicircle DNA Vector Free of Plasmid Bacterial Sequences and Capable of Persistent Transgene Expression *In Vivo*

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ABSTRACT

We have shown previously that minicircle DNA vectors free of plasmid bacterial DNA sequences are capable of persistent high level of transgene expression *in vivo*. The minicircle is generated in bacteria from a parental plasmid containing an inducible phage ϕC31 integrase gene and a therapeutic expression cassette flanked with *att*B and *att*P sites. The ϕC31-mediated intramolecular recombination between *att*B and *att*P results in the formation of two circular DNA molecules, one containing the eukaryotic expression cassette (minicircle), and the other the plasmid bacterial DNA backbone (BB). Previously, the minicircle was purified away from the plasmid BB by a restriction enzyme digestion step and ultracentrifugation in cesium chloride. We have now included the endonuclease I-*Sce*I gene together with its recognition site in the minicircle-producing plasmid to allow the linearization and degradation of the plasmid BB in bacteria. The minicircle can then be isolated by routine plasmid purification procedures such as a one-step affinity column. With additional modifications to our previous strategy, we can prepare a minicircle encoding a 4-kb human factor IX expression cassette, up to 1.8 mg of minicircle with 97% purity was prepared from a 1 liter bacterial culture. The high yield, simple purification, and robust and persistent transgene expression make these vectors viable for gene therapy applications.

OVERVIEW SUMMARY

Minicircle DNAs devoid of plasmid bacterial sequences are superior to standard plasmid as a nonviral DNA vector because: (1) of its relative safety due to the reduction of the inflammatory unmethylated CpG motifs; (2) more efficient transgene expression due to a reduced size; and (3) more robust and persistent transgene expression. The development of a one-step purification technology to isolate minicircle directly from bacterial lysate using commercially available affinity column allows production of this robust DNA vector capable of meeting clinical requirements, making it useful for clinical applications.

INTRODUCTION

A DESIRABLE FEATURE of nonviral vectors for human gene therapy is their capability to express a sustainable and high level of therapeutic transgene products. We demonstrated previously that minicircle DNA vector free of plasmid bacterial DNA backbone (BB) is devoid of a silencing effect, and can express a high level of therapeutic protein indefinitely *in vivo* (Chen *et al*., 2003). Minicircles delivered into mouse liver could express up to 12 μg of human factor IX (hFIX) per milliliter of serum, more than twice the wild-type concentration, from mouse liver for up to 7 weeks. These results suggest that minicircle can serve as ideal nonviral DNA vectors for gene ther-

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apy. Minicircles were first designed because we had found that plasmid BB, including the plasmid origins of replication, the F1 phage origin of replication, and the antibiotic resistance gene, silenced transgene expression when these DNA elements were covalently connected to the expression cassette (Chen *et al*., 2004). An increase in transgene expression could be achieved by using a purified expression cassette, or by simply cutting twice through the plasmid BB before delivery, allowing dissociation of the expression cassette from the plasmid bacterial DNA in mouse liver (Chen *et al*., 2001). The silencing effect was dependent on the expression cassette being covalently connected with plasmid bacterial DNA sequences but varied in an unpredictable manner between expression cassettes and bacterial backbones. The molecular mechanism underlying the plasmid bacterial DNA-silencing effect is not well understood, although the CpG dinucleotides, which are enriched in bacteria-derived DNA sequences (Paillard, 1999; Chen *et al*., 2004), have been suggested as the responsible DNA component (Qin *et al*., 1997; Schwartz *et al*., 1997; Krieg *et al*., 1998; Li *et al*., 1999; Tan *et al*., 1999; Yew *et al*., 2002). Significant enhancement in transgene expression has been achieved by elimination of the CpG motifs in the plasmids (Yew *et al*., 2000, 2002; Hodges *et al*., 2004). We decided to develop a minicircle production technology to completely and conveniently remove these problematic DNA elements from the vector.

In addition to overcoming a silencing effect, the size reduction in some systems may contribute to the high and sustainable level of transgene expression related to more efficient transfection. Removal of the approximately 3-kb bacterial sequence may greatly improve vector availability by increasing the efficiency in diffusion, cellular, and nuclear entry of the transgene. This has been suggested by other groups as the mechanism responsible for the robust transgene expression by minicircle in other tissues such as mouse skeletal muscle and human head and neck carcinoma grafts in nude mice (Darquet *et al*., 1997, 1999). Zhang and colleagues (2004), and Molnar and co-workers (Molnar *et al*., 2004) have demonstrated that vector size has a substantial inverse impact on transgene expression level when transfected into mouse muscle *in vivo*, or delivered to HeLa cell *in vitro*. With hydrodynamic transfection into liver, transfection efficiencies do not appear to play a significant role in transfection efficiencies because our results show similar levels of DNA in liver by Southern blot and *in situ* hybridization regardless of the vector makeup (Chen *et al*., 2001). A similar result was also demonstrated by Zhang *et al*. (2004).

The minicircle production technology we developed was based on the *Streptomyces* temperate phage ϕC31 integrase-mediated intramolecular recombination (Chen *et al*., 2003). We constructed the minicircle producing parent plasmid by including the ϕC31 gene under the control of the inducible BAD promoter, together with a transgene expression cassette flanked with phage attachment sites *att*B and *att*P in the same plasmid. The ϕC31-mediated intracellular recombination between *att*B and *att*P was induced in *Escherichia coli* Top 10 by the BAD promoter inducer L-arabinose. This results in two circular DNA molecules: one is the transgene expression cassette with a 36-bp hybrid *att*R, termed the minicircle, and the second is the circular plasmid bacterial backbone containing the 37-bp hybrid *att*L (BB). In our previous study, the minicircle was produced by a two-step procedure. The two episomal DNA circles were copurified by passage through an affinity column, and then BB plasmid was linearized by restriction digestion, and the minicircle was separated by ultracentrifugation in cesium chloride. A major disadvantage of the original method was the relatively low yields of ~100 to 200 μg of minicircle from 1 liter of overnight bacterial culture. In addition, restriction enzyme linearization of the BB was costly, and the cesium chloride banding step was labor intensive. To overcome these problems, we have developed a new minicircle production technology that allows one-step purification of the minicircle DNA vector from bacteria, using a

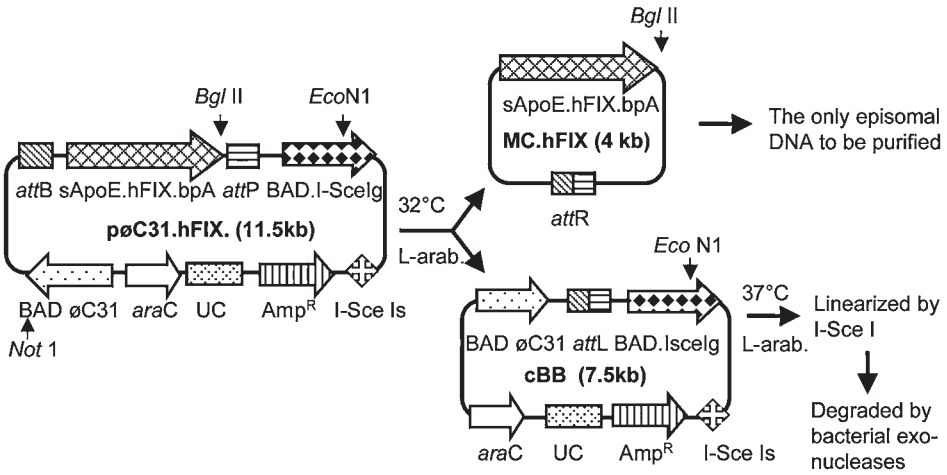

FIG. 1. Generation of minicircle and destruction of cBB. pϕC31.hFIX was the minicircle-producing plasmid, which produced two DNA circles, MC.hFIX and cBB, via ϕC31-mediated intramolecular recombination. sApoE, enhancer-promoter composed of the apolipoprotein E hepatic locus control region (sApoE.HCR) and human α_1-antitrypsin (hAAT) promoter; hFIX, human factor IX cDNA with a truncated form of its first intron (Miao *et al*., 2000, 2001); MC, minicircle; cBB, plasmid bacterial backbone circle; *att*B, bacterial attachment site; *att*P, phage attachment site; *att*L, left hybrid sequence; *att*R, right hybrid sequence; BAD, BAD promoter; *ara*C, *ara*C repressor; Amp^R, ampicillin resistance gene; UC, pUC plasmid replication origin; I-SceIg, I-*Sce*I gene; I-SceIs, I-*Sce*I cutting site; L-arab., L-arabinose.

commercially available affinity column. Using this technology, large quantities of relatively pure DNA were prepared suitable for use in gene transfer studies in animals.

MATERIALS AND METHODS

Vector construction

We inserted the intron-encoded endonuclease I-*Sce*I gene (amplified by polymerase chain reaction (PCR) from plasmid pCMV3xnls.IsceI) (Donoho *et al*., 1998) under the control of the BAD promoter, together with an I-*Sce*I cutting site (I-SceIs), downstream of the *att*P site in the plasmid pBAD.ϕC31.sApoE.hAATp.hFIX (Chen *et al*., 2003), resulting in the plasmid pϕC31.hFIX (Fig. 1). An additional copy of the BAD.ϕC31 expression cassette was inserted into the *Not*I site in tandem with the original one, resulting in the plasmid p2ϕC31.hFIX (Fig. 3A). We inserted one to three additional copies of BAD.ϕC31 into the *Not*I site in the construct pBAD.sApoE.hAATp.hFIX (Chen *et al*., 2003), and replaced the 4.0-kb sApoE.hAATp.hFIX.IntA.bpA expression cassette with the 1.2-kb expression cassette RSV.Kan^R.bpA, resulting in the plasmid pϕC31.Kan^R (Fig. 2A).

Production of minicircle

The previous protocol for minicircle production (Chen *et al*., 2003) was used to produce minicircle in this study with minor modifications. Briefly, overnight bacterial growth from a single colony of plasmid-transformed *Escherichia coli* Top 10 was spun down in a clinical centrifuge (model Allegra/6KR; Beckman Coulter, Fullerton, CA) at 20°C, 3000 rpm for 20 min. The pellet was resuspended 4:1 (v/v) in fresh LB broth containing 1% L-arabinose. The bacteria were incubated at 32°C with constant shaking at 250 rpm for 2 hr. After adding a one-half volume of fresh LB broth (pH 8.0) containing 1% L-arabinose to the culture, the incubator temperature was increased to 37°C and the incubation continued for an additional 2 hr. Episomal DNA circles were prepared from bacteria, using plasmid purification kits from Qiagen (Valencia, CA).

Determination of kinetics of minicircle production mediated by different copy numbers of BAD.ϕC31

The plasmid pϕC31.Kan^R (Fig. 2A) was used to produce minicircle encoding the RSV.Kan^R.bpA expression cassette according to the previous minicircle production protocol (Chen *et al*., 2003). During the incubation, a 15-ml aliquot of the concentrated bacteria was removed 0, 15, 30, and 60 min after the start of ϕC31 induction, and episomal circular DNA was prepared from bacteria by passage through an affinity column. One microgram of the resulting DNA was digested with *Alw*NI and separated by agarose gel electrophoresis.

Determination of kinetics of I-SceI-mediated destruction of cBB

The kinetics of cBB destruction were evaluated with plasmid p2ϕC31.hFIX (Fig. 3A) and the modified minicircle protocol described above. A 15-ml aliquot of concentrated bacteria was periodically removed, and episomal DNA was prepared with a Qiagen plasmid midi kit. Five hundred nanograms of the resulting DNA was digested with *Bgl*II and *Eco*NI before being loaded into each well. After separation by electrophoresis, all of the DNA bands were quantified with Quantity One software (Bio-Rad, Hercules, CA). Minicircle formation kinetics were expressed as the percent change in the 4-kb minicircle DNA band among all four DNA bands, whereas bacterial backbone destruction kinetics were expressed as the percentage of all the impure DNA, including the unrecombined parental plasmid, as represented by the 12.2- and 1.5-kb bands, together with the plasmid bacterial DNA, as represented by the 9.7-kb band. (Fig. 3C). To determine the quality of the resulting DNA, we performed agarose gel electrophoresis with 1 μg of the minicircle with or without digestion by *Bgl*II plus *Eco*NI before electrophoresis was performed.

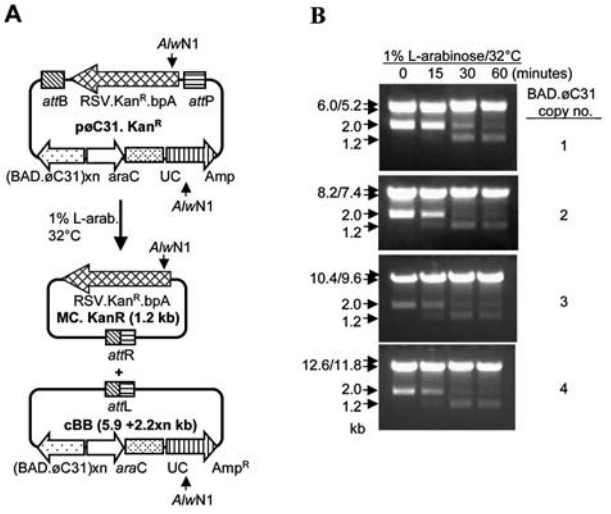

FIG. 2. Optimization of ϕC31 copy number. (**A**) Flow chart of minicircle production. pϕC31.Kan^R, the minicircle-producing plasmid with one to four copies of BAD.ϕC31; MC.Kan^R, the minicircle encoding RSV.Kan^R.bpA; RSV, Rous sarcoma virus long terminal repeat promoter; Kan^R, kanamycin resistance gene; bpA, bovine growth factor polyadenylation signal. (**B**) Kinetics of minicircle production with various copy numbers of BAD.ϕC31. Each lane contained 1 μg of DNA from bacteria collected at each time point and digested with *Alw*NI before electrophoresis. The size of the parental plasmids, containing one, two, three, or four copies of BAD.ϕC31, is 7.2, 9.4, 11.6, and 13.8 kb, respectively. The DNA signal at the top of each lane is from two DNA fragments, including the larger *Alw*NI-restricted fragment of the unrecombined parental plasmid, and the *Alw*NI-digested BB. Their size is variable according to the BAD.ϕC31 copy number in the parental plasmid. The 2.0-kb band is the short *Alw*NI fragment of the unrecombined plasmid, and the 1.2-kb band is the linearized MC.Kan^R.

RESULTS

Generation of minicircle and destruction of plasmid bacterial DNA circle

The principle of one-step minicircle purification technology was to destroy the bacterial plasmid DNA after recombinase-mediated recombination, leaving the minicircle expression cas-

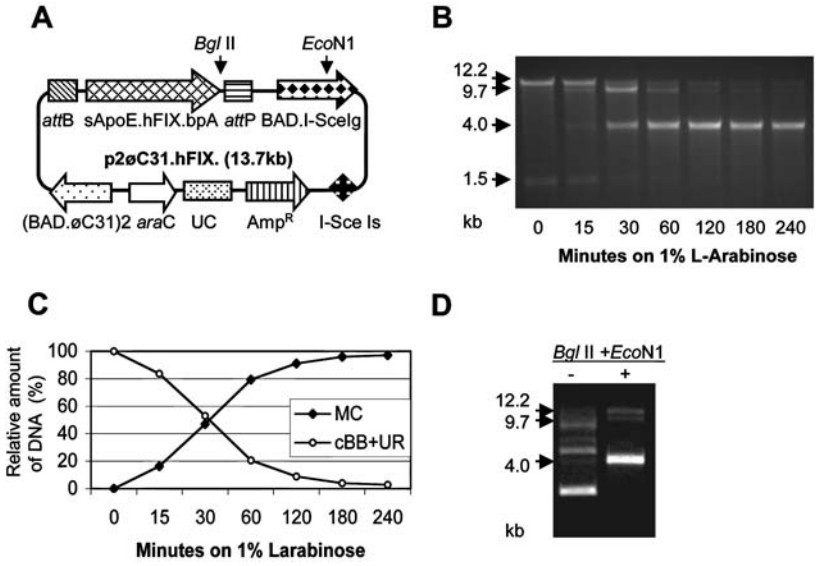

FIG. 3. Kinetics of minicircle generation and cBB destruction. (**A**) Scheme of the minicircle-producing parental plasmid. (BAD.ϕC31)2, two tandem copies of BAD.ϕC31. (**B**) Agarose gel showing the change in the intensity of DNA bands representing MC and impure DNA. Each lane contained 0.5 μg of *Bgl*II- and *Eco*NI-digested DNA. (**C**) Determination of the dynamic change of the minicircle product and impure DNA as visualized in the agarose gel (**B**). All four DNA bands in the agarose gel were quantified. Each determinant represents the percentage of MC or the impure DNA (cBB+UR) among all four bands. UR, unrecombined plasmid. (**D**) Characterization of minicircle. Each lane contains 1 μg of the episomal DNA at the end of the 4-hr incubation with (right lane) or without (left lane) digestion with *Bgl*II plus *Eco*NI.

sette as the only episomal DNA circle in the bacteria. This would allow isolation of circular expression cassettes by simple affinity chromatography. To do this, we placed the intron-encoded endonuclease gene, I-*Sce*I, under the control of the BAD promoter as we did with the ϕC31 gene, and added an I-*Sce*I recognition sequence outside the *att*B and *att*P sites, into plasmid pBAD.ϕC31.sApoE.hFIX (Chen *et al*., 2003), resulting in the plasmid pϕC31.hFIX (Fig. 1). I-*Sce*I was chosen because it cuts an 18-bp recognition sequence that is not present in the *Escherichia coli* genome. When the bacteria were incubated in the presence of the inducer, 1% L-arabinose, two reactions in the minicircle-producing plasmid would occur. The first reaction was the ϕC31-mediated intramolecular recombination between *att*B and *att*P, which resulted in the formation of two DNA circles, the minicircle and plasmid bacterial DNA circle. The second was the linearization of the plasmid bacterial DNA circle by I-*Sce*I cleavage of the I-*Sce*I site, followed by degradation of the linearized DNA by bacterial exonucleases (Benzinger *et al*., 1975), leaving the minicircle as the only episomal DNA in the bacteria. This episome could then be purified from bacteria by affinity column chromatography, as for any routine plasmid DNA. We digested the resulting DNA with *Bgl*II and *Eco*NI before loading it onto the gel, and found a predominant 4-kb band, representing the minicircle, and three weak bands 10.0, 7.5, and 1.5 kb in size. The 10.0- and 1.5-kb bands were derived from unrecombined minicircle-producing plasmid, and the 7.5-kb band from plasmid BB circle (data not shown). These results confirmed the formation of minicircle and destruction of most of the plasmid bacterial backbone.

Optimization of ϕC31 copy number

The endonuclease I-*Sce*I was produced at the same time as ϕC31, which potentially resulted in a decrease in the yield of minicircle DNA vector. Thus, we conducted experiments to determine whether multiple copies of BAD.ϕC31 in a plasmid could increase the efficiency of minicircle formation. To do this, one to three additional copies of the BAD.I-SceIϕC31 were inserted into the ϕC31.hFIX (Fig. 1), and the sApoE.HCR.hAAT.hFIX+IntA.bpA expression cassette was replaced with an RSV.Kan^R.bpA cassette, resulting in pϕC31.Kan^R (Fig. 2A). We used the above-described protocol to generate the minicircle encoding the RSV.Kan^R.bpA expression cassette. Aliquots of DNA isolated from the bacterial cultures were analyzed by gel electrophoresis (Fig. 2B). We found that the best result was produced from the construct with two copies of BAD.ϕC31, which produced much more minicircle DNA, as represented by the 1.2-kb band, than the plasmid containing only one copy of BAD.ϕC31 at 15 min. Minicircle formation was not further increased in the plasmid containing three or four copies of ϕC31 expression cassette (Fig. 2B).

Optimization of minicircle production protocol

According to the observation described above, we placed a second copy of BAD.ϕC31 into the plasmid pϕC31.hFIX (Fig. 1) in tandem with the original one, resulting in the plasmid p2ϕC31.hFIX (Fig. 3A). To determine the optimal conditions for production of minicircle and destruction of plasmid BB, we tried different broth compositions and incubation temperatures. We found that the addition of a half-volume of fresh LB broth (pH 8.0) containing 1% L-arabinose to the bacteria 2 hr after the start of induction and an increase in the temperature from 32 to 37°C resulted in more consistent BB plasmid destruction. We determined the kinetics of minicircle formation and plasmid BB destruction, using the optimized plasmid and modified protocol. Aliquots of bacteria were removed periodically, and

the episomal DNA was prepared with a Qiagen plasmid kit. Five hundred nanograms of episomal DNA was digested with *Bgl*II plus *Eco*NI, and separated in an agarose gel. As expected, four DNA bands were observed (Fig. 3B), the strong 4-kb band representing the minicircle, the weaker 12.2- and 1.5-kb bands from unrecombined parental plasmid, and the 9.7-kb band representing the plasmid BB. We quantified all DNA bands with Quantity One from Bio-Rad. The change in the formation of minicircle and destruction of plasmid BB is presented as the percent change of each form of DNA in Fig. 3C. Consistent with our previous observation, the majority of ϕC31-mediated intramolecular recombination occurred within the first 30 min after the addition of L-arabinose and progressed to near completion within 60 min. This was determined by monitoring the increasing intensity of the 4-kb minicircle band, which was much stronger at 30 min compared with the 15-min time point, and was maximized at 60 min. The destruction of plasmid BB was apparent by the decrease in intensity of the 9.7-kb band, which occurred more slowly. Both the 12.2- and 9.7-kb bands became less intense over time, and the 2.0-kb band was undetectable by 120 min (Fig. 3B). We found that by 240 min after L-arabinose induction, contaminating plasmid BB and unrecombined parental plasmid were reduced to 3% of total plasmid DNA. We examined the quality of the resulting minicircle by comparing the agarose gel migration pattern of cut and uncut minicircle. The 4.0-kb linear minicircle band, which was the most predominant DNA signal in the digested sample, was barely visible in the uncut sample, suggesting that most of the minicircle was supercoiled with small amounts of the nicked form (Fig. 3D). When 16 μg of minicircle encoding the sApoE.hFIX expression cassette prepared by this one-step purification procedure was infused into mouse liver, using the hydrodynamic procedure, more than 10 μg of human factor IX per milliliter of serum was expressed over a period of 7 weeks, a transgene expression profile similar to that of minicircle prepared by the cesium chloride banding protocol (Chen *et al*., 2003) (data not shown). We therefore used this as our standard protocol for one-step purification of minicircle DNA vector, and consistently produced 1.0 to 1.8 mg of this 4-kb minicircle from 1.0 liter of overnight bacterial growth with more than 95% purity.

DISCUSSION

We have previously demonstrated that minicircle DNA vectors can express robust levels of therapeutic transgenes, making them more useful for nonviral vectors for human gene therapy (Chen *et al*., 2003). The one-step purification technology developed in this study has greatly improved the production procedure, and overcome several limitations with our previous protocol. First, the yield is greatly increased, with as much as 1.8 mg of 97% pure supercoiled minicircle from 1 liter of bacterial culture. This is 9-fold more minicircle compared with the original protocol. Second, the costly restriction digestion step and the time- and labor-intensive cesium chloride banding step have been eliminated. Consequently, the cost and the time for production of the same amount of minicircle is only a fraction of that described in the original protocol. Third, the mutagen ethidium bromide is avoided in the procedure. This work establishes a scalable method that results in highly purified minicircle DNA vector suitable for gene therapy applications.

The present one-step minicircle preparation technology consists of a plasmid in which both the recombinase ϕC31 and the endonuclease I-*Sce*I are placed under the control of the same BAD promoter, and are produced at the same time in each bacterial cell. A potential problem relates to the fact that I-*Sce*I could destroy the minicircle producing parental plasmid before the minicircle is formed, resulting in a decrease in minicircle yield. We took three measures to alleviate this problem. First, we inserted two copies of BAD.ϕC31, determined to be the optimal copy number to obtain the desired intramolecular recombination. Second, we incubated the bacteria at 32°C, which was optimal for ϕC31 intramolecular recombination (Groth *et al*., 2000) but not for I-*Sce*I endonuclease activity. At this temperature, the destruction of unrecombined parental plasmid was minimized (Monteilhet *et al*., 1990). Third, we prepared the initial broth according to a composition known to favor ϕC31 activity. It has been demonstrated that I-*Sce*I activity is optimal at reaction with higher pH (Monteilhet *et al*., 1990). We added one half-volume of fresh LB broth (pH 8.0), and increased the incubation temperature to 37°C to increase the I-*Sce*I cutting activity 2 hr after L-arabinose induction, presumably after most of the recombination had occurred. As a result, the loss of unrecombined parental plasmid was minimal, as indicated by the high yield of minicircle. The parental plasmid is 13.7 kb in size, 3.4-fold the size of the 4.0-kb minicircle. Therefore, the minicircle yield of 1.0 to 1.8 mg equals 3.4 to 6.0 mg of the parental full-size plasmid per liter, a molar yield similar to standard protocols used to prepare this parental plasmid.

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