Minicircle DNA Technology

MNxxxx-1

User Manual

Please see PAC for storage temperatures

Version 8
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Contents

Product Description

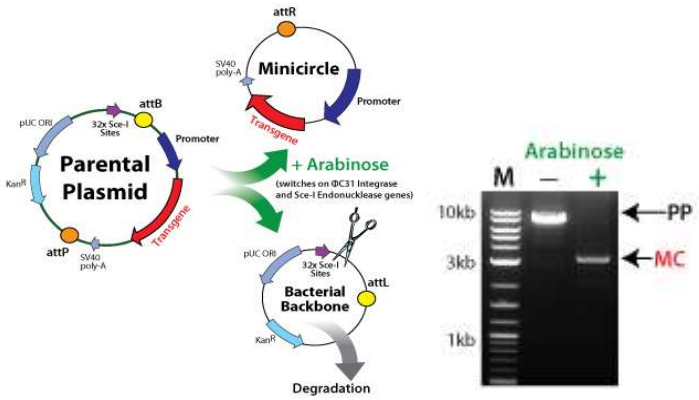
Minicircle Technology

Minicircles (MC) are circular DNA elements that no longer contain antibiotic resistance markers or the bacterial origin of replication. These small vectors can be used in vivo or in vitro and provide for long-term transient expression of one or more transgenes without the risk of immunogenic responses that can be caused by the bacterial backbone in standard plasmids.

Production of minicircles requires a special parental plasmid (PP) and an engineered E. coli strain that allows both propagation of the parental plasmid and production of the minicircles. Minicircles are conditionally generated by the expression of an inducible ΦC31 integrase via intramolecular (cis-) recombination. The full-size MC-DNA construct is grown in a special host E. coli bacterial strain that harbors an arabinose-inducible system to express the ΦC31 integrase and the I-SceI endonuclease simultaneously. The ΦC31 integrase produces the MC-DNA molecules from the full-size PP-DNA upon arabinose induction. The PP-DNA contains a number of engineered I-SceI restriction sites that are subject to I-SceI endonuclease digestion and ultimate destruction of the PP-DNA. The MC-DNA lacks I-SceI restriction site so that it remains intact. The 32 copies of I-SceI sites in the PP-DNA enables production of superclean MC-DNA without PP-DNA contamination. This bacterial strain produces purified MC-DNA in a time-frame and quantity similar to those of routine plasmid DNA preparation.

Minicircle DNA vectors allow sustained transgene expression in quiescent cells and tissues. These vectors have demonstrated 10- to 1,000-fold enhancement with an increase in long-term transgene expression compared to regular plasmids in quiescent tissues *in vivo* and *in vitro*. The mechanism of enhanced transgene expression may result from eliminating heterochromatin formation induced by the plasmid backbone and methylation and transgene silencing.

The major obstacle to the widespread use of mincircles has been the time-consuming, labor-intensive production. The MC-Easy™ System from SBI enables a simple, reproducible and efficient way to produce high quality Minicircle DNA. The finely tuned growth and induction media produces minicircle DNA that is free of parental and genomic DNA contamination. The kit also includes an additional, innovative method for degrading any contaminating genomic DNA using an ATP-dependent DNAse reagent that will selectively remove genomic DNA but will not affect minicircle DNA yield. This method produces clean and effective minicircle every time.



ZYCY10P3S2T *E.coli*

ZYCY10P3S2T *E.coli* Minicircle producer competent cells have been prepared and tested by a modification of the procedure published in Nature Biotechnology by Kay et al. (Kay MA, He CY, Chen ZY. 2010, Nature Biotechnology). These cells are suitable for the cloning of minicircle-based plasmids. ZYCY10P3S2T *E.coli* minicircle producer cells are derived from a BW27783 bacterial strain that stably express a set of inducible minicricle-assembly enzymes, ΦC31 integrase and I-SceI homing endonuclease. This bacterial strain produces purified minicircles in a time frame and quantity similar to those of routine plasmid DNA preparation, making it feasible to use minincircles in place of plasmids in mammalian transgene expression studies.

Note: Other competent *E. coli* strains cannot be substituted for minicircle production as they do not have the appropriate genome modifications to produce minicircles.

List of Components

Please refer to the PAC of the product received for the list of components.

Storage

Please refer to the PAC for the specific product(s) you purchased for the proper shipping and storage conditions. Separate products in described in this protocol require different storage conditions.

Protocols

cDNA Cloning into Minicircle Parental Plasmids

1) Clone your gene-of-interest into the parental plasmid of choice either by using the multiple cloning site, or by using SBI's Cold Fusion cloning kit (Cat #MC010, MC100, or MC101).

2) Your insert should contain a Kozak sequence, ATG start codon, and stop codon.

3) Proceed to transformation of ZYCY10P3S2T E. coli cells.

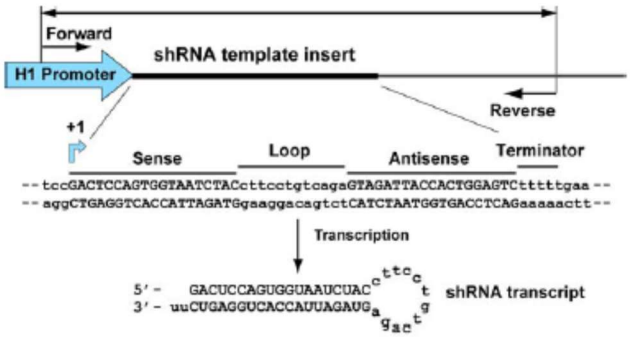
Cloning into Minicircle shRNA Parental Plasmids

The Minicircle shRNA parental plasmids contain either a CMV or EF1 promoter that drive expression of GFP or GFP and Puromycin resistance, followed by an H1 promoter that drives expression of the shRNA construct. Typically, 3-5 target sequences for the gene of interest need to be selected and tested to identify functional shRNAs with at least 70% silencing efficiency of the target mRNA. Although there is no standard rule for selecting the target mRNA binding sites for shRNA sequences, we have found the following criteria useful:

- 19-29 nt in length, usually longer oligos (25-27 nt) are more robust and give better silencing efficiencies however 19 nt oligos could be also used.
- Unique sequences with less than 70% homology with other mRNA sequences in a RefSeq database. Especially avoid homology of the central portion of the shRNA with other non-target mRNA sequences. Flanking sequences usually tolerate mismatches without reduction in silencing efficiency.
- 40-55% GC content
- No more than 4 consecutive A's or T's.
- No more than 5 consecutive G's or C's

• No thermodynamically stable secondary structure (0 Kcal/mol)

• A 5' terminus (3-5 flanking nucleotides) on the antisense strand should be more AT-rich than the 3' terminus.



The template sequences coding for the shRNA targeted to each selected target site must contain both the sense and anti-sense strand, and be designed to form a stem-loop structure when transcribed. In addition, both the top and bottom strands of the entire shRNA sequence (sense-loop-antisense-terminator) must be synthesized and annealed to make a double-stranded DNA sequence that can be cloned into the vector. The features of the oligonucleotides coding for the shRNA template sequence should include the following:

• The 19-29 nucleotide sense and antisense mRNA sequences. Usually longer siRNAs (25-27 nt) have better silencing efficiencies although 19-nt oligos are more commonly used.

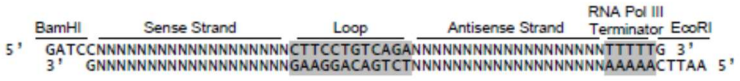
• A hairpin loop sequence between sense and antisense portion. The 9-nt loop sequence (5'-TTCAAGAGA-3') is most commonly used in RNA silencing experiments, but we have used a 12-nt sequence (5'-CTTCCTGTCAGA-3'), which generates similar results. Loop sequences of 3 to 15 nucleotides have been used successfully by different investigators.

• A TTTTT terminator sequence for RNA polymerase III.

• A BamHI and EcoRI restriction site overhang sequences for directional cloning of annealed shRNA template oligonucleotides into the vector.

• Using of initiation G nucleotide in the first position of sense portion of SHRNA is not necessary, as RNA polymerase III could initiate transcription from any +1 nucleotide of the H1 promoter.

The top and bottom strands of the shRNA template oligonucleotides should be designed to look like the following diagram after annealing:



1) Linearize the MNSI vector with EcoRI/ BamHI. Digest the plasmid overnight at 37°C. Then purify the plasmid DNA.

2) Anneal the shRNA template oligonucleotides

a. Use regular non-phosphorylated oligos. Dissolve the shRNA template oligonucleotides in an appropriate amount of deionized water to a final concentration of 20 µM.

b. Set annealing reactions for each experimental shRNA template

1 µl Top strand shRNA template oligo
1 µl Bottom strand shRNA template oligo
18 µl 10 mM Tris-HCl, pH 8.5
20 µl Total Volume

c. Heat the reaction mix to 95°C for 2 min in a thermocycler.

d. Turn off the thermocycler and let it cool to room temperature over 20 minutes.

3) Ligate the shRNA template into the linearized minicircle shRNA parental plasmid.

a. Set up a ligation reaction for each shRNA template

1 µl linearized vector
1 µl annealed double-stranded shRNA template
1 µl 10x T4 DNA ligase buffer
6 µl deionized water
1 µl T4 DNA ligase (40 U/ µl)
10 µl Total Volume

b. Incubate the ligation reaction at 16°C overnight.

4) Proceed to Transformation of of ZYCY10P3S2T E. coli

Minicircle Production Protocol using the MC-Easy™ Kit

Transformation of ZYCY10P3S2T E. coli Minicircle producer strain

1) Thaw competent cells on ice.

2) Add DNA from ligation reaction, using 1-5 µl of the reaction to one vial of ZYCY10P3S2T cells gently rotating the pipette tip through the cells while dispensing. Gently tap tubes to mix.

3) Alternatively, for empty parental plasmid transformation add 20-100 ng DNA to the vial.

4) Incubate cells on ice for 30 minutes.

5) Heat-shock cells for 30 seconds in a 42°C water bath. Do not shake.

6) Place cells on ice for 2 minutes.

Recovery of ZYCY10P3S2T E. coli

This procedure is very important for Kanamycin resistant constructs.

1) Add 0.2 ml of room temperature S.O.C. Medium to the vial. Transfer to a bacterial culture tube.

2) For tubes containing ligation reactions, shake at 250 rpm (30°C or 37°C) for 90 minutes.

3) For tubes containing empty parental plasmid DNA: shake at 250 rpm (30°C or 37°C) for 60 minutes.

Plating and Mini-Preps of ZYCY10P3S2T E. coli

1) Pre-warm culture plates to 37°C. Chilled plates will decrease ZYCY10P3S2T E coli transformation efficiency.

2) Spread 50-200 µl of bacterial solution on an LB plate containing 50 µg/ml kanamycin. Incubate overnight at 37°C.

3) Keep the remaining solution at room temperature, and spread onto a new plate the next day in the event no colonies are seen.

4) Pick 3-5 colonies to grow in 2 ml of LB containing 50 µg/ ml kanamycin. Grow overnight and then extract the plasmid by miniprep.

5) Check the Minicircle parental plasmid by restriction digest analysis and sequencing.

6) If the parental plasmid looks correct, make glycerol stocks from the miniprep bacterial culture and store them at -80°C. You are now ready to produce Minicircle DNA from the parental plasmid DNA.

Growth and Induction of the Minicircle Producer Strain

The following steps describe production of Minicircle DNA from the parental Minicircle plasmid which was produced in the previous steps.

Day 1: Inoculation

1) On day 1, inoculate 2 ml of LB containing 50 µg/mL kanamycin with a single colony. Incubate at 30°C shaking at 250 rpm for 4-6 hours. And then inoculate 0.5-1 ml to 200 ml of 1X growth medium. Put back into incubator at 30°C shaking at 250 rpm for overnight.

2) If the bacteria is in glycerol stock, take one and loop directly put into 200ml of 1X growth medium.

Note: The bacteria must be fresh. Bacteria in LB agar + kanamycin plates stored at 4°C should not be used beyond 5 days. Glycerol stocks can be kept in -80°C for several years. It is recommended that overnight cultures are not grown for more than 16 hours. Using older bacteria will increase the chances of parental plasmid and genomic DNA contamination.

Note: The ratio of flask size to culture volume at 5:1 (vol:vol) will ensure proper bacteria growth.

Day 2: Induction

1) After overnight growth, measure the pH and OD600 of the culture medium. The pH should be around 7 and the OD600 should be 4-6. **Record the OD600 and the pH in your laboratory notebook in case you need to troubleshoot later.**

a. If the OD600 > 8 and the pH < 6.5, we recommend restarting the protocol from the beginning. Check the temperature and ventilation of the incubator.
b. If the OD600 is between 6 and 8 then proceed to the step 3.

2) If the OD600 is 4-6, combine 200 ml of the overnight culture with 200 ml of 1X Induction medium.

3) If the OD600 is between 6-8, combine 200 ml of the overnight culture with 400 ml of 1X Induction medium.

4) Incubate the mixture again at 30°C, shaking at 250 rpm for 3 hours, and then increase the temperature to 37°C for 1 hour. Total induction time is 4 hours (Longer induction time may increase bacterial death and cause genomic DNA contamination).

5) Take 1 ml of the bacterial culture and do a miniprep followed by restriction digest analysis to check the quality of minicircle plasmid. Once the quality of the plasmid is confirmed via miniprep, you may proceed with a maxiprep.

6) Pellet bacterial at 4°C, and store the pellet at -20°C or -80°C.

Day 3: Extraction of Minicircle plasmid

1) Follow protocol of Invitrogen's PureLink HiPure Plasmid Purification Kit making sure to use (2X) the volume of R3, L7, and N3 buffer for each pellet.

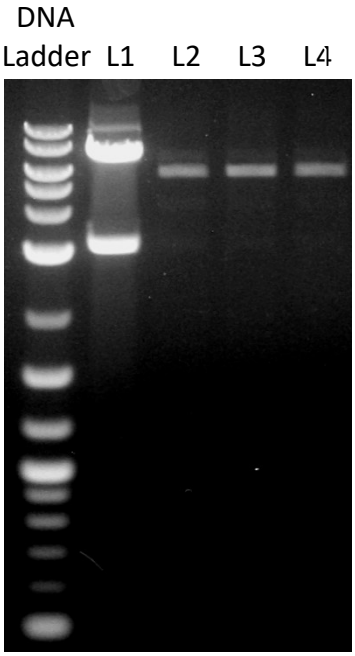
2) Alternatively, use the QIAGEN maxiPrep Kit protocol using (2X) the volume of P1, P2 and P3 Buffers.

3) Digest 0.5-1.0 µg of minicircle plasmid with restriction enzyme and then run on a 1.5% agarose gel to ensure plasmid quality.

4) If the quality is good, go to the transfection procedure offered in Section I to check the function.

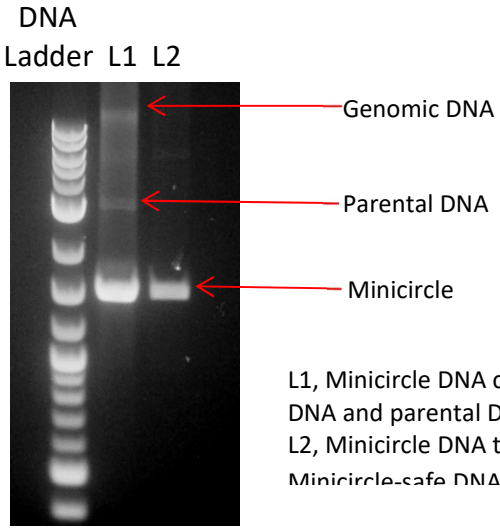
5) If the minicircle preparation contains genomic DNA or parental DNA or both, continue with the next steps to remove the parental and genomic DNA contamination.

a. If the parental DNA contamination is more than 10% higher than the minicircle DNA yield, you must re-start again. Please refer to the troubleshooting section for more details.
b. It is okay to proceed with excess genomic DNA contamination (in the absence of parental DNA contamination).



This is an example of a purified minicircle plasmid that does not have any genomic or parental plasmid contamination. If your preparation looks like this, you can proceed by transfecting the minicircles into your cells-of-interest.

L1, Parental DNA of SRM100PA-1 cut by Mfe1. **L2, L3, L4**, Minicircle DNA of SRM100PA-1 cut by Mfe1.



L1, Minicircle DNA contaminated with genomic DNA and parental DNA. L2, purified minicircle L2, Minicircle DNA treated with Nde1 and Minicircle-safe DNAse L3

This is an example of a minicircle plasmid that has both genomic DNA and parental plasmid contamination (L1). If your minicircle preparation looks like this, you will need to perform the Removal of Genomic and Parental Plasmid DNA contamination steps before transfecting it into your cells-of-interest.

Removal of Genomic and Parental Plasmid DNA contamination

1) Linearize parental DNA with 1 or 2 restriction enzymes that cut the bacterial backbone only without cutting inside the minicircle DNA. Using plasmid editor software will ensure you choose the appropriate restriction enzymes.

2) Use Minicircle-safe DNase (included) to digest the linearized parental DNA and genomic DNA, according to the protocol below.

~400 µl plasmid (use the whole amount you obtained in the last step)

20 µl 25 mM ATP
50 µl 10X Reaction Buffer
20 µl DNase
~10 µl water
500 µl Total Reaction Volume

Incubate at 37°C for 2-16 hours

3) Inactivate DNase by incubation at 70°C for 30 minutes.

4) Digest 1-2 µl from the DNase-treated and untreated samples using same restriction enzyme and run on an agarose gel to check the results. If the reaction was successful, you should see minicircle DNA only (there will be no parental plasmid or genomic DNA contamination in the sample).

Re-precipitation of minicircle DNA and Removal of DNase and Restriction Enzymes

1) Add 1/10 volume of Precipitation Buffer to the DNase-treated solution and mix by pipetting.

2) Add 2 volume of 100% ethanol or 0.7 volume of 100% Isopropanol. Mix by inverting the tube and you can see the white precipitate forms.

3) Put on ice for 30 min.

4) Centrifuge at 15000x g for 15 minutes. Carefully discard supernatant. Do not disturb the DNA pellet.

5) Wash pellet with 1ml of 70% Ethanol, centrifuge 5 minutes at 15,000 X g.

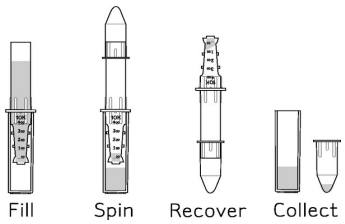
6) Discard the supernatant, leaving the pellet in the tube to air dry for 5 minutes.

7) Dissolve plasmid with 1-2 ml of DNAse free water.

8) Optional: Continue to next step using 2ml DNA Centrifugal column to remove dNTPs and salts. Alternatively, if ATP and dNTPs do not influence your downstream experiment, you can stop here.

Optional: Removal of ATP and dNTPs with 2ml DNA Centrifugal columns

2ml DNA Centrifugal columns can be used to remove dNTPs, primers, other macromolecular components, salts and buffers.



1) Insert the 2ml DNA Centrifugal filter into a filtrate collection tube.

2) Add up to 2 ml of sample to the device and cover with concentrate collection tube.

3) Place filter device into the centrifuge rotor and counterbalance with a similar device.

a. For a swinging bucket rotor, spin at 1,000x g for 30 min or 3000x g for 10 min.
b. For a fixed angle rotor, spin at 7,500 × g for 10-30 min.

4) Wash step: add 2 ml of the buffer you want and repeat spin procedure.

5) Remove the assembled device from the centrifuge and separate the 2ml DNA Centrifugal column from the filtrate collection tube.

6) To recover the concentrated solute, invert the filter column to concentrate collection tube. Spin for 2 minutes at 300-1,000 × g to transfer the concentrated sample from the column to the tube. NOTE: For optimal recovery, perform the reverse spin immediately.

7) Measure plasmid concentration and check A260/A280 ratio. The A260/A280 ratio should be 1.8-1.9.

Transfect Minicircle DNA with MC-Fection™ to validate the construct

1) Seed 1.5X10^5 target cells/ ml per well in a 12-well plate. SBI uses 293T cells, but you can use any target cells that you prefer.

2) Incubate overnight at 37°C. Cells should be 50%-70% confluent the next day.

3) In a sterile Eppendorf tube add the following (per well):

100 µl DMEM (without FBS)
1 µg Minicircle DNA
2.5 µl MC-Fection™

4) Mix by vortexing vigorously for a few seconds. Incubate at room temperature for 15 min.

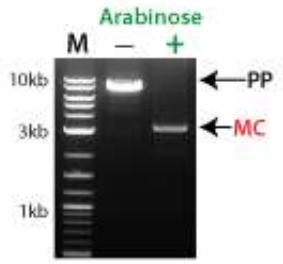
5) Add the mixture to one well of a 12-well plate and incubate at 37°C, 5% CO2 overnight.

6) Check for fluorescent reporter protein expression (e.g. GFP or RFP), or other marker gene, or protein-of-interest expression.

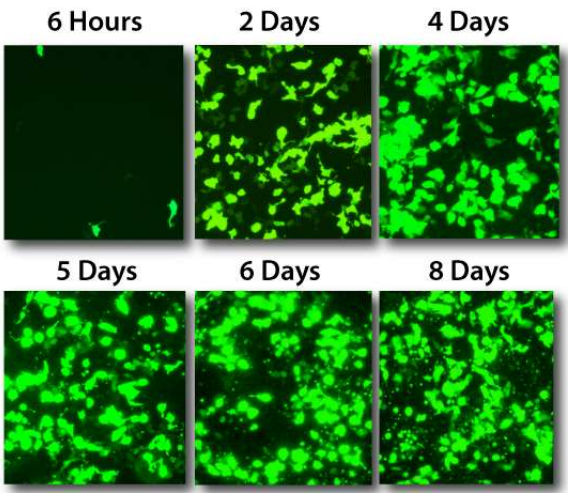
Example Data and Applications

Tail Vein injection of Minicircles for in vivo expression of transgenes

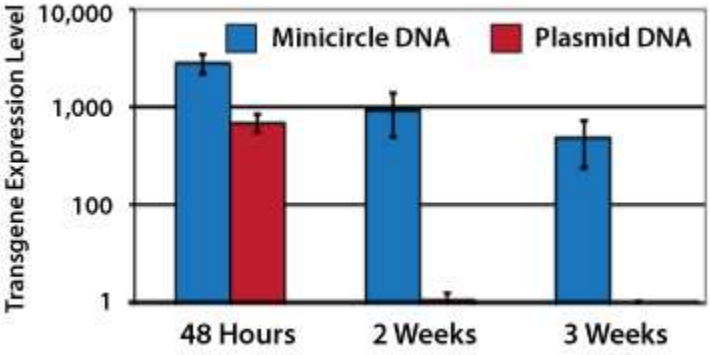
Minicircle DNA can be injected into mice by tail vein injection for in vivo expression of transgenes. We recommend following the protocols set up by your institution's animal handling facility.



The gel image here shows uninduced (minus arabinose) and induced (plus arabinose) ZYCY10P3S2T *E. coli*. The resulting minicircles (~3kb) are produced.



Transfection of 1 µg minicircle DNA (pMC.CMV-MCS0EF1-GFP-SV40PolyA) into HEK293 cells. Expression of GFP persists for at least 8 days.



Example data of *in vivo* transgene expression in a mouse injected with minicircle DNA through tail-vein injection. Plasmid DNA is degraded after 2 weeks of expression, while minicircle DNA expression remains strong for at least 3 weeks.

Troubleshooting

The most common problems in minicircle production are:

- Genomic DNA contamination
- Parental DNA contamination
- Low yield

Problems	Possible causes	Solutions
Genomic DNA contamination	Bacteria over growth.	Shorten culture time and reduce the amount of innoculum. Check ventilation and shake speed.
	Bacteria is not fresh or was stored at 4˚C for > 1 week.	Always use fresh bacteria.
	Ventilation is not good.	Keep the ratio of flask size to culture volume at 5:1 (vol:vol).
	Culture medium pH is too low or incubation time is too long.	Keep the culture pH >6.5.
	Mechanical damage to bacteria, such as vigorously vortexing after freezing.	Resuspend bacteria pellet carefully and completely after freezing by gently vortexing and pipetting up and down.
	Lysis time > 5min.	Invert bottles gently after adding lysis buffer and keep at RT <5min.
	Insufficient quantity of SDS in lysis buffer to permit complete binding to the cellular debris.	When the buffer is stored at temperatures below 20°C, which causes the SDS to precipitate out of solution. Warm Lysis buffer at 50^0C a few minutes to dissolve the SDS completely.
	Solution was not mixed soon enough after the addition of buffer P3.	This could result in the formation of clusters of PDS (potassium dodecyl sulfate) together with cellular debris including genomic DNA. These clusters have a tendency to float. If these floating pieces are added to a column then genomic DNA contamination almost definitely will occur. Mix immediately after adding Buffer P3 (or N3, or S3) by inverting vigorously 4-6 times. Remove floating pieces by filter. Do not overload the column beyond its capacity.
Parental DNA contamination	Bacteria is stored at 4˚C for more than one week.	Always use fresh bacteria.
	pH <6.5 before induction.	Check pH and correct to >6.5 before induction.
	Cold induction medium or temperature during induction is too low.	Use room-temperature induction medium.
	Induction time is not enough or too long.	Adjust induction time.
	>10% parental plasmid contamination	Start the protocol from the beginning.

Low yield	The plasmid failed to precipitate, which is especially common when the size of minicircle DNA <3.5 kb.	Use high speed centrifuge 15000g for 30-60 min to precipitate small size minicircle DNA.
	Bacteria was not resuspended or lysed completely.	Use double amount of resuspension buffer, lysis buffer and neutralization buffer. Consider adding LyseBlue reagent to resuspension buffer. Solution should turn evenly blue after adding lysis buffer and inverting 4-5 times.

Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site: **http://www.systembio.com**

For additional information or technical assistance, please call or email us at:

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Licensing and Warranty Statement

Limited Use License

Use of the Minicircle Technology™ (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

The purchaser of the Product is granted a limited license to use the Product under the following terms and conditions:

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- The Product may not be resold, modified for resale, or used to manufacture commercial products without prior written consent of SBI.
- This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research.

SBI has pending patent applications related to the Product. For information concerning licenses for commercial use, contact SBI.

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SBI warrants that the Product meets the specifications described in this manual. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or provide the purchaser with a refund. This

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