SBI

SBINSIGHTS

Minicircles: The Next Generation of Plasmids

Produce Your Own Minicircles with the MC-Easy Minicircle Production kit

The Development of Minicircles, Interview with Inventor Mark Kay, M.D., Ph.D

Issue.
05

ACCELERATING DISCOVERIES THROUGH INNOVATIONS

mRNAExpress™

mRNA Synthesis Kit

Only From SBI System Biosciences

Transcribe and Deliver mRNA into Cells for Rapid Expression

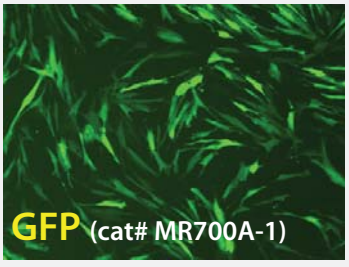
Generate Highly Concentrated Synthetic mRNAs

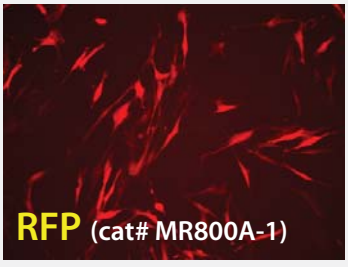
SBI's mRNAExpress™ mRNA Synthesis Kit is designed for *in vitro* transcription of mRNAs to be used for transfection of mammalian cells, oocyte micro-injections, *in vitro* translation and other applications. Each reaction can produce 20-40μg RNA. Positive control mRNAs for GFP and RFP as well as pre-made mRNAs for MyoD, Nanog, Oct4, Sox2, Lin28, Klf4 and c-Myc are also available.

Each mRNAExpress Kit comes complete with:

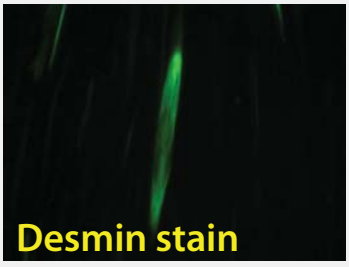
- pMRNA^{xp} T7 promoter, poly-A tail cloning vector
- Robust T7 RNA polymerase
- Anti-reverse ARCA cap analog
- Modified nucleotide triphosphates
- DNase & phosphatase
- RNA purification spin columns

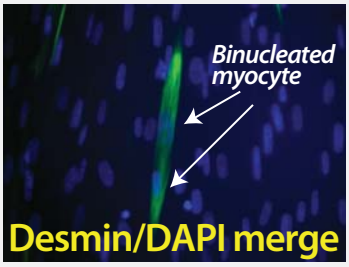
Positive Control mRNAs for GFP and RFP to Track your RNAFections





mRNAExpress MyoD mRNA (cat# MR900A-1) Transdifferentiates Skin into Myocytes





Find out more:

www.systembio.com/mrnaexpress

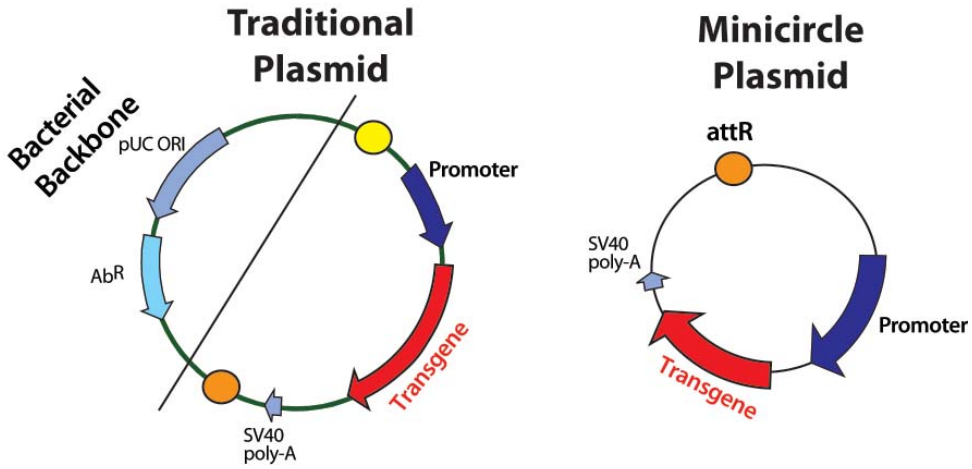
650-968-2200 • info@systembio.com

Minicircles: The Next Generation of Plasmids

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. Minicircles are typically expressed for a period of several weeks, without integrating into the host cell genome, thus preventing unwanted genomic changes in the cells.

Minicircle versus Traditional Plasmid Structure

Traditional plasmids have a bacterial backbone portion that contains the origin of replication and an antibiotic resistance gene, which are use for propagation of the plasmid in bacterial culture. Additionally, they have the necessary promoters, transgenes, and reporter genes used for expression of the transgene in the target cells. While the bacterial portions of the plasmid are necessary for propagation, they can cause immune reactions in the target cells which direct the plasmids to degradation pathways. The host cells basically recoginze the bacterial portion of the plasmids as "foreign" and thus destroy it, allowing for only a short expression time of the transgene. Some plasmids also contain a mammalian antibiotic selection marker which facilitates integration of the plasmid into the host cell genome, thus prolonging the expression of the transgene, but causing integration into random sites within the chromosome, which can cause unwanted changes in the host cell.

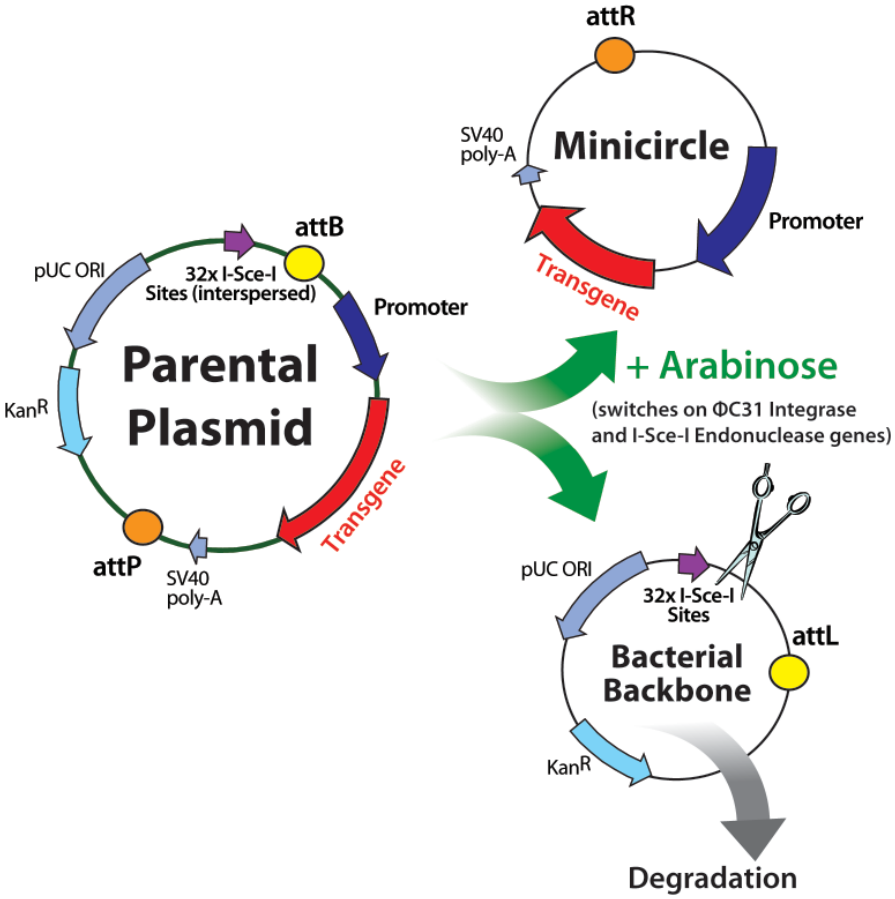


Minicircles are devoid of the bacterial portions of the plasmid. So while minicircles cannot replicate or integrate into the target cells, they are expressed for a longer period of time because there is no bacterial backbone directing the plasmids to a degradation pathway. Since they do not integrate into the host cell genome, there is no risk of them causing unwanted permanent changes in the cells resulting from integrating into random sites in the genome.

The Components of the Minicircle System

Production of minicircles requires a special parental plasmid (PP-DNA) and an engineered *E. coli* strain (ZYCY10P3S2T *E. coli*) that allows both propagation of the parental plasmid and the production of the minicircles. Minicircles are conditionally generated by an expression of inducible ΦC31 integrase via intramolecular (cis-) recombination.

The full-size PP-DNA construct is grown in ZYCY10P3S2T *E. coli* that harbor an arabinose-inducible system to express the ΦC31 integrase and the I-SceI endonuclease simultaneously. The ΦC31 integrase produces the MC-DNA molecules and bacterial backbone DNA from the full-size PP-DNA upon arabinose induction. The bacterial backbone contains a number of engineered I-SceI restriction sites that are subject to the digestion of I-SceI endonuclease and ultimate destruction of the bacterial backbone. The MC-DNA is lacking I-SceI restriction sites so that it remains intact. The 32 copies of I-SceI sites in the bacterial backbone secure the 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 Production

Minicircle production involves the following steps:

1) Cloning into the Parental Plasmid-of-choice

2) Transformation of the ZYCY10P3S2T *E. coli* cells

3) Growth of the ZYCY10P3S2T *E. coli* cells to propagate the Parental Plasmid

4) Induction of the ZYCY10P3S2T *E. coli* cells to produce the minicircles

5) Purification of the minicircles

6) Analysis of the produced minicircles on an agarose gel.

The complete protocol takes about 3-4 days from start to finish.

Minicircle production can be acheived by using reagents that you make in the lab yourself according to the protocol published in: Mark A. Kay, Cheng-Yi He & Zhi-Ying Chen. A robust system for production of minicircle DNA vectors. Nature Biotechnology, (2010). doi:10.1038/nbt.1708.

Alternatively, SBI has developed the MC-Easy Kit which is an all-in-one system that allows you to produce minicircles, purify them, analyze their purity, and clean up the preparation if necessary.

The produced minicircles can then be transfected into cells or injected into animals for *in vivo* expression of cDNAs, shRNAs, or microRNAs.

Common Problems with Production of Minicircles

The most common problems with minicircle production are low yield and contamination with parental plasmid or *E. coli* genomic DNA. These problems can be overcome by slightly modifying specific steps in the protocol. The yield of produced minicircles is inversely proportional to the size of the minicircle particles. Larger minicircles provide a higher yield, while smaller minicircles give a lower yield. SBI's MC-Easy Minicircle Production kit is a great way to easily produce your own minicircles.

Producing Minicircles using SBI's MC-Easy™ Kit

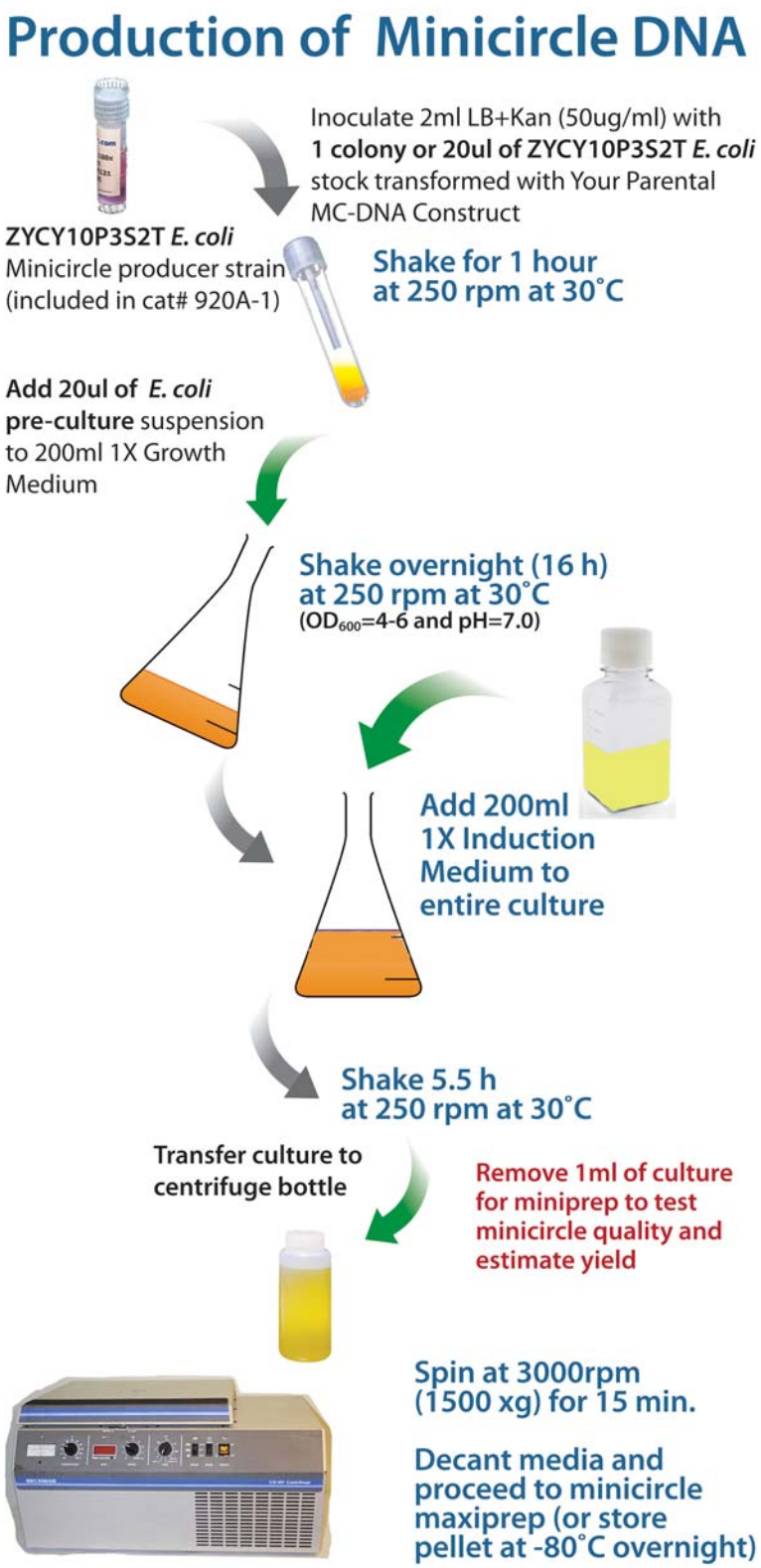
The MC-Easy system enables the simple, reproducible and efficient way to produce high quality Minicircle DNA for your experiments. The MC-Easy kit contains all of the reagents you will need to produce minicircles effectively.

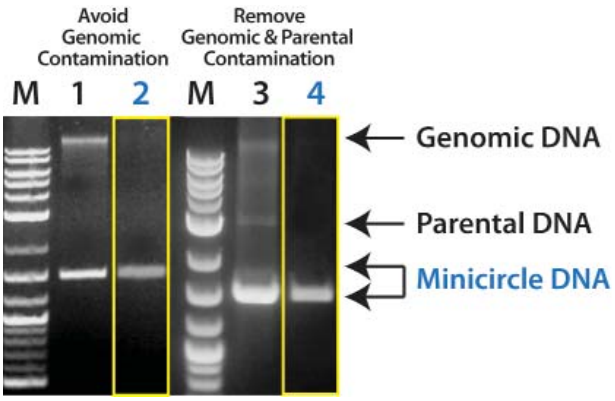
Start by cloning your insert into one of SBI's minicircle parental plasmids and transforming the ZYCY10P3S2T *E. coli* cells (available with the MC-Easy kit, or as a separate product). After selecting and validating a colony, innoculate the Growth Medium and grow overnight. The protocol indicates at what steps to measure the OD600 and the pH, which are critical to the success of the protocol.

The next day, add the Induction Medium to the culture and grow for 5.5 hours. The induction step enables the minicircles to form and the residual parental plasmid backbone to be degraded.

At this point, take an aliquot of the culture and perform a miniprep and restriction digest on it. When you run the agarose gel, you will want to check for genomic or parental plasmid contamination. It is important to do the restriction digest with 1-2 restriction enzymes because the minicircle parental plasmids are often supercoiled, which causes them to run at the incorrect size on a gel. Linearizing them enables you to detect the accurate size, and distinguish between minicircles, parental plasmid, and genomic DNA. Genomic DNA will run > 10 kb on a gel. The parental plasmid DNA will run at the size of the empty vector plus the size of the insert you added to it. The minicircles will run near size of the insert.

If there is no contamination of parental plasmid or genomic DNA, you can proceed with a Maxi or Mega plasmid preparation step to purify the minicircles.





If there is <10 % contaminating parental plasmid or genomic DNA, you can proceed by using the plasmid-safe DNase. After purifying the DNA from the minicircle preparation, use a restriction enzyme that cuts in the parental plasmid backbone (and not in the minicircle region). This will linearize the parental plasmid preferentially. Treating with the plasmid-safe DNase will degrade the linearized parental plasmid and genomic DNA, while preserving the minicircle DNA preferentially. After the DNase treatment, you will need to recover the DNA by ethanol precipitation. The figure at the left shows an example of when it is okay to use the plasmid-safe DNase treatment to clean up the minicircle preparations. Lane 1 shows genomic DNA contamination. Lane 2 shows the cleaned-up minicircle preparation after the DNase treatment. Lane 3 shows both genomic DNA and parental plasmid contamination. Lane 4 shows the cleaned-up minicircle preparation.

If there is >10% parental plasmid or genomic DNA contamination, you would want to start the minicircle production protocol over and make sure that the growth conditions are optimized. The MC-Easy Kit user manual has a troubleshooting section that indicates which parts of the protocol may need optimization depending on the type of contamination you are experiencing.

Benefits of MC-Easy versus your own reagents

The benefit of using the MC-Easy Minicircle Production kit is that the Growth and Induction Media have been standardized and tested for each lot. Before MC-Easy was available, many minicircle customers had difficulty with the different recipes for the Growth and Induction Media. The MC-Easy kit has standardized the reagents, by carefully controlling the pH, antibiotic, and induction agent within the media. All you have to think about is using the right size flask and whether your incubator is calibrated to the proper temperature. SBI also spent a significant amount of time and effort developing the minicircle clean-up steps. While the yield of minicircles may be slightly lower after the clean-up steps, these steps are less time consuming that starting the entire protocol over from the beginning.

Other Tips from SBI's Technical Support Team

It is important to follow the modified Maxi/ Mega plasmid purification steps recommended in the MC-Easy kit. The bacterial pellet will be quite large after such a long incubation. Therefore, complete resuspension and lysis of the pellet is key to getting the highest yield possible. However, incubation in the lysis buffer for too long can also lead to increased genomic DNA contamination.

We also recommend checking the pH and OD600 at the recommended points within the MC-Easy protocol, and keeping a note of them within your laboratory notebook. If you need help troubleshooting, these numbers will be key in determining what part of the protocol needs adjustment.

Typical Yield of Minicircle DNA

For a minicircle that is > 4 kb, the typical yield is about 0.5 mg. For a minicircle that is between 3-4 kb, the typical yield is about 0.25 mg. Minicircles < 1.5-3 kb will yield about 0.125 mg. The repurification steps usually result in 15-25% loss of product.

The Development of Minicircles

Interview with Mark Kay, M.D., Ph.D., Stanford University

How did you first come up with the idea of minicircles?

The concept behind the minicircle vectors came from unrelated studies starting in the 1990s when we were trying to unravel the molecular steps involved in how single-stranded recombinant adenoassociated viral (rAAV) vector genomes were converted to double-stranded DNA once inside the cell of a primary tissue. The rAAV proviral genomes were converted to double-stranded monomeric and concatemeric episomes. The puzzling finding was the discordance in transgene expression from an AAV proviral episome and plasmid DNA, which for all intents and purposes contain the same expression cassette, and only differ by short viral ITRs and bacterial backbone, respectively. The rAAV episomes resulted in relatively stable life-long transgene expression (in the absence of cell division) whereas plasmid-mediated transgene expression decreased by orders of magnitude over a few weeks.

We found that purified linear dsDNAs containing only an expression cassette or rAAV proviral genome containing the same expression cassette were both converted to circular and concatemeric episomes in tissues and provided similar robust and persistent transgene expression. Expression was silenced only with covalent linkage of the plasmid DNA bacterial backbone to the ends of the expression cassette. All of the data to date suggest that transgene silencing occurs at the level of transcription. Dr. Zhi-Ying Chen, a research scientist in the lab came up with a strategy to produce minicircles using a prokaryotic inducible recombinase system that allowed for the production of minicircle DNAs. This process was much simpler compared to other technologies that were available at the time, which involved isolating DNA fragments from gels followed by ligation *in vitro*. However, at this point the minicircle production system, while still more labor intensive compared to routine plasmid preparations, for the first time allowed large quantities of minicircle DNA vectors to be produced and robustly tested in small and large mammals. These studies provided the proof of concept on how useful these vectors would be and why we continued to pursue both improving production and unraveling the mechanisms involved in their enhanced gene expression profiles.

What were some of the challenges you faced while developing minicircle technology?

One of the problems we faced was that the arabinose induction used to promote the formation of minicircles never went to completion and at most we could get 80 to 90% minicircle purity without the inclusion of a CsCl based ultracentrifugation step--- a step most laboratory workers like to avoid. Dr. Chen tried to vary the production protocol to get additional purity but was unsuccessful. Then we learned that the endogenous L- arabinose transporter underwent an "all or none" response, which basically meant that there was always a population of bacterial cells in which the transporter was not expressed. This was overcome by using a different promoter to drive expression of the major arabinose transporter and in addition supply a second but different arabinose transporter gene. As a result, using the parental minicircle both improved yield and purity (over 99%) was achieved. However, in addition we thought that if minicircles were to be widely accepted—we needed to make the procedure nearly as simple as routine plasmid preparations.

We next pursued removing the inducible bacterial genes from the parental plasmid and placing an optimal number of these genes into the host bacterial strain used to propagate the minicircle vector. In the end it turned out that a lot of bacterial genome manipulation was needed and making all of these stable genetic manipulations turned out to be more difficult than we originally imagined. But over the next couple of years, Zhi-Ying Chen working with Chen-Yi He successfully produced the bacterial cell line, ZYCY10P3S2T. The bacterial cell name was derived from initials of their creator and designates the specific copy numbers of some of the genes added to the bacterial genome.

The creation of this particular bacterial cell line simplified the whole minicircle process! The parental plasmid construct is greatly simplified because all of the recombination induction genes and regulatory sequences were removed, making it a basic plasmid where the expression cassette of interest is inserted into a multiple cloning site between two short recombination sequences. Importantly the new system increased minicircle yield to amounts similar to a plasmid, provides >99% minicircle product in most cases, and most importantly made the labor and time involved required to very close to what is required for a preparing a regular plasmid vector.

Why do minicircles provide enhanced transgene expression?

The mechanisms involved in why there is more persistent transgene expression from quiescent cells is one that we have studied for years. We continue to do so. The lack of a bacterial plasmid backbone does decrease the concentration of CpG motifs, which when coupled with a lipid transfection reagent can exacerbate cytokine/innate immune responses. However, in our test model, the liver we use hydrodynamic transfection that does not require the use of lipids. We have found that CpG content and/or DNA methylation does not affect silencing. Using whole tissue ChIP analyses, we found that the minicircle and plasmid DNAs were chromatinized in cells. Moreover, we found different histone marks associated with persistent vs silenced plasmid DNAs. However, a causal effect has yet to be determined. We have recently used various lengths of random DNA sequences to replace the bacterial backbone DNA and determined that the length rather than sequence of the exogenous DNA outside of the expression cassette is the most important variable that affects silencing. High throughput nucleosome and ChIP seq studies are currently underway to further study the mechanism of plasmid-mediated gene silencing.

What is the best way to introduce minicircles into target cells?

In cells that divide, minicircle DNA will be lost as rapidly as DNA plasmids. That does not mean the minicircles might not be more useful even in dividing cells. For example, we found that minicircle DNAs expressing reprogramming factors are much better than routine plasmids for iPS cell induction. This might be due to the fact that lipids and minicircles have less pleotropic effects because they contain fewer CpG motifs but this is just a hypothesis. We have not used the minicircle in other tissues but any method of transfecting DNA should work. The smaller size of minicircles vs their corresponding plasmid could provide a transfection advantage in some situations but the general rule is that any DNA transfection method can be used with the minicircle vectors.

Can you give us a little history of you and your research interests?

I did an M.D.-Ph.D. at Case Western Reserve University and then did a Residency in Pediatrics and then a fellowship in Medical Genetics and Biochemical Genetics. During my years as a post-doctoral fellow, I started my work in the area of gene therapy. In 1993 I took my first faculty position at the University of Washington before moving to Stanford University in 1998. My lab is currently interested in gene/RNAi therapeutics and non-coding RNA biology. My group co-led the first clinical trials using rAAV vectors as a systemically administered vector for hemophilia B. We are interested in developing minicircle DNA vectors for clinical use but in terms of systemic administration, the limiting factor has been the inability to develop a clinically relevant delivery method (e.g. nanoparticle). We are happy to consider collaborations with those who are developing such technologies. The lab website is: http://kaylab.stanford.edu.

Meet Zhihong Sun, M.D.

Dr. Sun is a Research Associate in our Production and Custom Services group and has been working here since 2009

Where did you do your training?

I graduated from Suzhou University Medical school in China, with an M.D.

How did you get interested in science and research?

I first became interested in science and research in my earlier life when I was involved in several clinical research studies. I was the first doctor to perform an invasive heart electrophysiology study and radiofrequency ablation procedure to cure severe arrhythmias in children at Wuhan Children's Hospital (the biggest hospital in the center of China). Later, I received a grant from the Finland International Science Development Foundation, and worked at the Helsinki University Hospital Heart Research Institute. There I published three papers on Long QT syndrome and sudden cardiac death. When I came to the USA, I became interested in biotechnology work and came to SBI in 2009.

What do you like about working at SBI?

SBI's products and services are cutting-edge. I get to learn the latest information that basic researchers are studying and make an impact helping to develop new products and services. I've also been able to broaden my basic laboratory skills. SBI provides a stable work environment that is also less stressful than being a cardiologist.

At SBI I have been able to develop some new products and kits. The most recent product I developed was the MC-Easy Kit. I started with the original protocol published in Mark A. Kay, Cheng-Yi He & Zhi-Ying Chen. A robust system for production of minicircle DNA vectors. Nature Biotechnology, (2010). doi:10.1038/nbt.1708, but found a lot a variability between preparations. By standardizing the transformation recovery steps, media recipes, and DNase procedure, I was able to get more consistent results. I thought my newer protocol would be helpful to other researchers, so I developed the kit.

We want to hear from you!

Would you like to be featured in SBI's next newsletter? Send us a description of your work , what products or services are your favorite from SBI, and some photos. We will review your submission and contact you when we are ready to feature you and your lab!

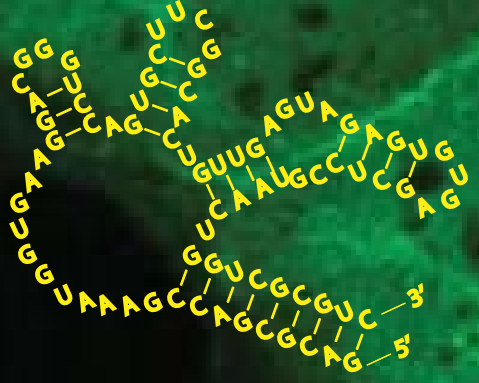
SPINACH

RNA Mimic of GFP

View RNA in Living Cells

Trafficking Dynamics

RNA Localization



Find out more:

www.systembio.com/spinach

650-968-2200 • info@systembio.com

SBI

System Biosciences

TECHNOLOGY FROM: Jeremy S. Paige, *et al.* RNA Mimics of Green Fluorescent Protein Science 333, 642 (2011).

UPCOMING EVENTS

2012 NIH Spring Research Festival, Bethesda, MD
April 25 - 26, 2012: SBI will be exhibiting. Please refer to the NIH Research Festival website for more details.

Stem Cell Industry Symposium, Bio-Trac/NIH CRM, Bethesda, MD
May 14, 2012:

LIVE WEBINARS

Several live webinars will be held between April and June 2012. Please check the SBI website for a full list of live and recorded webinars.

http://www.systembio.com/company/news-events/events/webinars

Would you like to hear from SBI?

Did you know that SBI can give your lab a customized webinar for your department, lab meeting, or other meeting? Let our Technical Support team know that you are interested and we can arrange for a presentation tailored to your interests. We can also offer on-site presentations, seminars, and occasionally hands-on training. Contact tech@systembio.com for more information.

System Biosciences, Inc.
265 North Whisman Rd.
Mountain View, CA 94043

Toll Free: 888.266.5066
Fax: 650-968-2277
Email: info@systembio.com
www.systembio.com