Apr 17, 2020

MiniCircle Production

Eric Danner[1]

[1]Max Delbrück Center

1 Works for me dx.doi.org/10.17504/protocols.io.7kwhkxe

Eric Danner
Max Delbrück Center

ABSTRACT

This is a cheap and efficent way to create Minicircles.

This MiniCircle System was originally published: Nat Biotechnol. 2010 Dec; 28(12): 1287–1289.

The cells and plasmid is available from SBI : https://www.systembio.com/wp-content/uploads/Minicircle-production-manual.pdf . However the company kit requires you to recurently buy supplies. So we bought the kit once and then were able to make indefinite preps.

This is our modifications drawn from from this paper :https://doi.org/10.1089/hum.2017.136 . This requires the MC E.coli (ZYCY10P3S2T). This protocol requires you to make your bacteria chemically competent yourself and this protocol does not discuss how to make them.

GUIDELINES

1. For TB-Medium: Phosphate tends to bind and precipitate at high temperatures to metals. So it is best to make the Tryptone/Yeast solution and the 10x phosphate buffer solution separate. Then autoclave, and mix together right before use. Discard the media if you see precipitation.
2. Minicricle Prep: I got better results using an endonuclease free maxi-kit that a normal maxi kit.
3. When cloning the plasmid use a normal E.coli strain as the MC E.coli ZYCY10P3S2T transforms less well. Use the MC E.Coli only for minicirlce production.
4. The minicircle backbone is a low copy number plasmid so you will get very little plasmid if you treat it light a high copy number plasmid!

MATERIALS

NAME	CATALOG #	VENDOR
Sodium Hydroxide	BP359500	Fisher Scientific
L-Arabinose	AB0071L.SIZE.100g	Bio Basic Inc.
Monopotassium phosphate	P9791	Sigma Aldrich
Potassium phosohate dibasic	60356	Sigma – Aldrich
Liquid LB medium		
Plasmid-Safe ATP-Dependent DNase Exonuclease	E3101K	Epicentre
EZ Clip wound closure	EZC KIT	Braintree Scientific
Kanamycin	K22000-25.0	Research Products International (rpi)
NucleoBond Xtra Midi EF plasmid purification kit	740420.10	Macherey and Nagel

Citation: Eric Danner (04/17/2020). MiniCircle Production. https://dx.doi.org/10.17504/protocols.io.7kwhkxe

NAME	CATALOG #	VENDOR
MC-Easy™ Minicircle DNA Production Kit (with Competent Cells)	MN920A-1	System Biosciences
Bacto™ Tryptone	#211705	BD Biosciences
Yeast Extract	2363.1	Carl Roth
NucleoSpin® Gel and PCR Clean-up	740609.10	Macherey and Nagel

Clone Stuff into your minicircle backbone

1 **Cloning**
The backbone has a low-copy plasmid ORI. So you need to get a larger volume for mini-preps than usual.

For sequencing one side of the multiple cloning sequence has a repetitive sequences for SecI cleavage and so can mess up the primer binding.
You can Sanger Sequence from 5' end with : 5'-tgatggtcgagactcagcgg-3'
or from the 3' end with 5'-tcgccttcttgacgagttcttct-3'

Use normal D5Halpha E-coli (or whatever you use) with Kan conditions for cloning as the ZYCY10P3S2T E.Coli strain transforms badly. Kan is toxic so requires 30-60 min shaking for resistance after heat-shock. Can use normal LB/kan media

Re-transform into ZYCY10P3S2T

2 Use 1-20ng of correct plasmid to re-transform into ZYCY10P3S2T cells. Preparation of these cells is the same as you would do for any chemically competent E.Coli strain.

These cells can be grown in TB media overnight and then frozen in a 50% glycerol stock.

Grow Culture

3 **Prepare your Terrific Broth** Media. These cells can grown under LB/Kan but for MC production you should use TB.

1) in 900ml: 12g tryptone, 24g yeast, 4ml glycerol.
2) 10x phosphate buffer: 23.12g KH_2PO_4, 125.41g K_2HPO_4 brought to final vol 1L.
Autoclave both bottles and then mix (9:1). Check for precipitation in bottom after months.
Add normal amount Kan (25-50ug/ml).

4 **Inoculate** your bacteria/glycerol stab or picked colony in 2ml of TB for 2 hours. 37C, 250 rpm.

Add 100ul to 200ml. Ideally pre-warmed TB w/Kan. Shake 220-250 rpm for 16 hr. (you don't want things to overgrow so check earlier than 16 hr).

I have also just added a frozen glycerol stab to cold or warm TB/kan and things worked fine.

5 **Check OD600**. *The biggest issue to be careful of is bacteria overgrowth as it results in bacteria death and release of endotoxins, non-recombined Minicircle plasmid,s, gDNA.* You want an OD600 of 4-6. Look at the SBI protocol for more information.

OD600 measurement (1:10 dilution with TB) - 1:10 dilution will give 0.4 - 0.6 which you then multiply by 10 to get original OD600.

-If the OD600 >8.5 then throw out cells and do again also if pH is <6.5 - this is a bad sign according to SBI. Otherwise you get too much gDNA contamination. 4-6 give the least gDNA contamination.

Citation: Eric Danner (04/17/2020). MiniCircle Production. https://dx.doi.org/10.17504/protocols.io.7kwhkxe

6 **Prepare induction medium**: 200ml LB + 200µl 20% arabinose (no kanamycin necessary).
Mix 200ml induction media with 200ml culture.

Check pH and titrate to about pH 7

Now you may need to add NaOH to bring the pH to 7-7.3.

7 **MC induction:** Take the 200ml culture + 200ml induction mix (check pH and adjust with 1N NaOH to 6.5 - 7) . Or 400ml induction media as noted above.

Arabanose activates the recombinase and also activates a nuclease to destory the DNA sequence that has the portions of the plasmid we are not interested in.

32 ° C, 250rpm, 3 - 5 hours. (no harm in letting it go to 5 hr)
You can check with a mini (1-2ml) every hour to see the plasmid recombine. Mini prep is linearized with restriction enzyme and run on a gel. Lower band is recombined minicircle, upper band is entire plasmid.

8 **Pellet and Maxi:** pellet the rest of the culture at 4 ° C and use Maxiprep (if necessary: store the pellet at -20C)
The plasmid is low copy, you can pellet up to 400ml (400 ml before the addition of the induction media) cells for OD600 = 6 in the MN Xtra Kit. In the maxi-kit it has a formula based on the OD600 and states how much lysis buffer to add. It is important to get enough lysis buffer to lyse all the cells. You do not have to worry about having too much DNA binding the collumn, but rather that you dont get fullly lysed bacteria.

Elute in 150ul TB

Clean up MiniCircles

9 **linearize and run on gel.** Take an aliquot of the purified MC and run undigested on a gel. You can also add a digested aliquot that is cut in one spot.

You may see a smear at the very top showing the the bacteria gDNA. You will want this to get removed. Also you will likely see a band of the original plasmid size. This gives you an idea of how much plasmid/gDNA contamination you have.

10 **Cut plasmid backbone and gDNA with restriction enzymes**
Now we use restriction enzymes to cut up the plasmid that was not recombined in to a minicircle and also the gDNA.

Find 2 restriction sites that exist in the backbone but not in your finished mini-circle.

Digest:
Final 200µl volume: 20µl buffer, 5µl Restriction enzyme 1, 5µl Restriction enzyme 2, total eluted maxiprep. Fill to 200 ul with H2O). Put on shaker 1-2h 37 ° C.

11 **Digest the linear DNA:** Use plasmid-safe DNase to digest the linearized gDNA and plasmid. It will not digest the circular minicircle.

200 µl from restriction enzyme digestion + 25 µl 10x DNase buffe + 10 µl ATP + 5 µl H2O + 10 µl DNase.
37 ° C overnight (16 -20 hours) on shaker.
Plasimid safe protocl

Citation: Eric Danner (04/17/2020). MiniCircle Production. https://dx.doi.org/10.17504/protocols.io.7kwhkxe

12 **Re-purify**
Halt enzyme- 30min 70C

Use pcr/gel cleanup mini kit. Each colum can bind 25g. So you might have to use multiple columns in serie. Elute in 20ul TE or water after warming column up to 70C.

Check concentration with Nanodrop. Take an aliquot and run on a gel.

Citation: Eric Danner (04/17/2020). MiniCircle Production. https://dx.doi.org/10.17504/protocols.io.7kwhkxe