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Purification of S. meliloti replicons via PFGE

For methodology of pulsed field gel electrophoresis (PFGE) of Rm1021, I recommend the papers from Sobral's group (Sobral et al. 1991; Sobral et al. 1991; Honeycutt et al. 1993) . Sobral was unable to resolve the uncut replicons of R.meliloti except by using a TAFE apparatus (Beckman Instruments--no longer commercially available). We prepared our pSymA library from SwaI digested DNA. SwaI cuts pSymA once and the resulting DNA can be easily resolved from the other SwaI DNA fragments.

Preparation of agarose embedded DNA

We found that the Bacterial Plug kit made by Biorad works well. We use the following modifications:
1. I use more cells per ml/plugs. (about 5 times more than Biorad recommends) For 5-ml plugs--Inoculate 10 ml TY in a 125-ml Bellco flask with colony of Rm1021. Grow to about an OD600 = 1. Spin down 5 ml cells. Wash once in 5 ml T10E 1 pH 8 + 0.1% sarkosyl, then again in 5 ml $T_{10}E_1$. Resuspend in 2.5 ml cell suspension buffer, add 2.5 ml Clean Cut agarose (Biorad).
2. The lysozyme and proteinase K treatment steps are as per kit instructions except I use 50 mg lysozyme in 25 ml 1X lysozyme buffer and 10 mg proteinase K in 25 ml PK buffer. I rinse the plugs well with sterile water before addition of PK buffer and also after overnight digestion at 50°C with PK.
3. Washes are as described in kit instructions: 2- 1 hour washes each in 1x wash buffer, one wash for 1 hour in 1X wash buffer + 1mM PMSF (PMSF is light sensitive) Wash one more time in 1X wash for 1 hour, then in 0.1X wash buffer for 1 hour. Store in 0.1X wash buffer.
Recipes from Biorad:
Lysozyme reaction buffer is proprietary. I haven't tried substituting any published buffers
Proteinase K reaction buffer pH 8: 100 mM ETDA, 10mM Tris, 1% N Lauryl sarcosine, 0.2% sodium deoxycholate
Wash buffer: 10 mM Tris pH 8, 50mM EDTA

Restriction digestion of agarose-embedded DNA

As per Biorad protocol

Casting the gel

We cast the gel in the tray as per manufacturers instructions and use 1% pulsed field certified agarose (Biorad). This agarose works much better than their more expensive "chromosomal grade agarose" for the size range in which we are interested. A useful tip I learned from our Biorad representative is to place the plug portions on the teeth of the comb and cast the agarose around the comb. For a 15 well comb (21 cm wide) I usually use 1/3 plug per tooth. If the plugs are not excessively moist when you place them on the comb and if the agarose is cool enough, the plugs will not float away. This is much less tedious than stuffing wells with fragile agarose! It also works well for the preparative gels we have been running. For a preparative gel, I usually cut the plugs in half lengthwise and place them end to end along the comb.

Running the gel

To resolve the 1.3-Mb SwaI band from the rest we use the following conditions:
Biorad CHEF DRIII
1% agarose gel and running buffer 1/2X TBE
14°C
ramped switch time from 60-120 seconds over 24 hours
120 ° angle
6 V/cm
DNA > 1.6 Mb is not resolved well under these conditions. The 1.8-Mb SwaI chromosomal band and the 1.7-Mb SwaI psymB band can be distinguished from each other but are not well separated. For preparative gels I trim the edges from the gel containing marker lanes and small amount of DNA, stain the edges with ethidium bromide, photograph with a ruler, then reassemble the gel and cut out the appropriate size band from the middle for additional purification. I have tried both electroelution and purification from low melt agarose. Currently I favor electroelution. The yield is about twice that obtained with low melt and the resulting DNA does not have contaminating agarose. However purification by electroelution takes longer because an additional CHEF run is required. I have wondered if it is possible to isolate DNA by electrophoresis onto DEAE membrane (Schleicher and Schull Corp) I have heard from those at the Stanford Genome Center that "others" have tried it with no success. Apparently, large DNA is impossible to elute from the membrane. However, I haven't talked to anyone who has tried this, so I don't know what conditions have been tested.

Purification of DNA from low melt agarose

After the first run with 1% pulsed field gel certified agarose the DNA must be electrophoresed again. This is necessary because DNA molecules of random size become tangled and comigrate with the large DNA. The band containing DNA is excised and flipped around 180° such that the leading edge is now the trailing edge and recast in 1% SeaPlaque GTG agarose (FMC Corp), 1/2X TBE. Run conditions are as before. The band is excised as before and sliced into 1 cm long pieces. See Gnirke et al. 1993 for similar protocol. The pieces are equilibrated 2 x 30 min in 1X beta-agarase buffer, melted at 65°C for 10 minutes, equilibrated at 40°C and b-agarase (NEB) is added. (I have been using about 1.5 units b-agarase per 100 microliters melted agarose.) Digest for 2 hours. Chill, spin to remove undigested agarose and concentrate using a Microcon-50. The yield will be very low-about 0.5 μg per gel. I am unsure why recovery is so poor-I estimate the amount of DNA present in the excised band after the first gel to be about 3 to 5 μg. beta-agarase treatment, like electroelution, shears the DNA, so purification of intact DNA requires special treatment (Maule et al. 1994) .

Purification of DNA by electroelution

The DNA must still be gel purified twice. However it is not necessary to used low melt agarose for the second gel. The band is excised and placed in Spectrapore dialysis tubing (either purchase high grade or boil in 1mM EDTA before using) At first, I tried to electroelute in a minigel box. Others have been able to electroelute 880-kb DNA this way. It did not work for my 1.6-Mb DNA-all the DNA stayed in the gel slice. Next, I tried electroeluting with a dialysis bag clamped and taped down in the CHEF DRIII using the same conditions as for the original gel, including the 24 hour run time. This seems to work fine. I have not tried altering run settings or decreasing the run time. Before removing buffer from dialysis bag, I massage the bag well. Buffer and bag rinses are concentrated in a Centricon-30.

References

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