DNA Fragment Isolation and Purification
For Microinjection in NOD Embryos

Equipment
QiaFilter plasmid Maxi-prep Kit (Qiagen, cat. # 12263)
Qiaquick Gel Extraction Kit (Qiagen, cat. # 28704)
Slide-A-Lyzer MINI Dialysis Units (3.5 K MWCO) plus float (Pierce, cat. # 69558)
Scalpel blade, clean

Reagents
Luria-Bertani Broth (LB)¹
Agarose²
Crystal Violet Stock (10 mg/ml)
Elution Buffer (10 mM Tris-HCl, pH 8.5)³
Microinjection Buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4)⁴

Notes:
1. Make sure final NaCl concentration is 10 g/l for most efficient plasmid DNA production.
2. Qbiogene QA-agarose™ gave the best recovery. Fisher agarose was bad for recovery.
3. Elution Buffer

   Stock Solution 1M Tris-HCl, pH 9-9.5 @ 25°C, 200 ml

   • 3.04 g Tris-HCl
   • 21.88 g Tris base
   • add Millipore ddH₂O to 200 ml
   • pH should be 9-9.5
   • filter sterilize through 0.22 um filter flask

For 100 ml of Elution Buffer

   • 1 ml Stock Solution (1M Tris-HCl, pH 9-9.5)
   • add about 90 ml Millipore dd H₂O
   • adjust pH to 8.5 with HCl (less than 1 drop conc. HCl)
   • bring to 100 ml with Millipore dd H₂O
   • filter sterilize through 0.22 um filter flask
   • divide into small aliquots
4. Microinjection Buffer

Stock Solution (1M Tris-HCl, pH 8.1-8.4)

- 16.08 g Tris-HCl
- 11.88 g Tris base
- add Millipore dd H$_2$O to 200 ml
- pH should be 8.1-8.4
- filter sterilize through 0.22 um filter flask

For 1L of Microinjection Buffer

- 10 ml Stock Solution (1M Tris-HCl, pH 8.1-8.4)
- 0.2 ml 0.5M EDTA pH 8.5 (commercial)
- add about 900 ml Millipore dd H$_2$O
- adjust pH to 7.4 with conc. HCl (about 6 drops)
- bring to 1000 ml with Millipore dd H$_2$O
- filter sterilize through a 0.22 um filter flask

General Considerations

It is essential to avoid traces of detergent. Use new sterile plasticware or thoroughly rinse any glassware that you use, such as the beaker for dialysis, and keep it dedicated for this purpose. Clean it yourself with water so that it does not get detergents through the regular institutional washing services. The 0.22 um filters should be prewashed with water prior to filtering Microinjection Buffer. Finally, the molecular weight cut-off of the dialysis unit will vary depending on the size of the construct.

Procedure

1. *Isolate large amounts of the corresponding plasmid DNA.* During the day (8 hrs), grow a fresh colony from a streaked plate with the selecting antibiotic in 3-5 ml LB + antibiotic. Then dilute the starter culture 1:500 to 1:1000 in two 1L flasks containing 250 ml LB + antibiotic culture. Grow overnight.
2. Purify the plasmid from culture according to the manufacturer’s instructions using the QiaFilter plasmid Maxi-prep Kit.
3. Resuspend the precipitated DNA in approximately 800 ul of Millipore dd H$_2$O (based on 500 ml of culture medium). This may vary depending on the copy number of your construct plasmid. The goal is to get the DNA as concentrated as possible prior to digestion.
4. *Digest plasmid DNA with appropriate enzymes.* You will need the enzymes that will excise the fragment to be microinjected.
5. Digest about 100-200 ug of plasmid DNA in a final volume of 200-400 ul. The digest efficiency can be increased if one half of the amount of enzymes is used for a 3-5 hr digest followed by addition of the remaining half of enzymes for overnight digestion.
6. Clean a small gel box, avoiding the use of soap but rinsing well with warm water and finishing with a dd H$_2$O rinse.
7. Prepare a 0.7-0.8% agarose TAE 1X gel with crystal violet 1:5000 (from 10 mg/ml stock). The percentage may vary according to the fragments to be separated but percentages higher than 1 percent can result in reduced recovery of DNA. Do not add EtBr. Use a thin comb, but combine several teeth with tape to get a larger single well (you will have to play with this to see how many wells you need to combine to load the 220-440 ul volume of digest plus loading buffer). Prepare the TAE 1X gel with autoclaved 50X TAE and Millipore dd H2O.

8. Add 1:10 loading buffer to the digest and load on gel. Run at 90 V for 2-3 hrs or until you get the maximum separation between the band of interest and the unwanted bands. It is usually useful to run 1.5 ug or more of a ladder. (With crystal violet, bands can be seen with normal light but the limit of detection is lower, so more ladder needs to be loaded. The advantage is that no UV light is required, so there is reduced UV-induced cleavage of the DNA).

9. **Collect the gel band and extract the DNA.** Cut out the band with a new clean scalpel blade and collect in a pre-weighed 50 ml conical tube. Calculate the agarose slice weight by difference. Process right away if possible.

10. Extract with the Qiaquick Gel Extraction kit, following the manufacturer’s instructions, but note:
    - Load the entire gel slice onto a single column, spinning for only 20 seconds each time you load 750 ul of the dissolved gel slice.
    - Do the additional QG buffer wash.
    - Make sure the PE buffer is reconstituted using Molecular Biology Grade ethanol.
    - Do not incubate during the PE wash, but go directly to centrifugation.
    - Elute with 50 ul of sterile home-made Elution Buffer (see above) or a dedicated new EB from the kit (filter sterilized) and warm it up to 50-60°C before adding onto the column. Then incubate for 5 minutes before spinning down.
    - Collect the eluate into a sterile tube.
    - Check the concentration with a spectrophotometer.

11. **Dialyze the Construct for Microinjection.** Dialyze 50 ul of construct for microinjection in 1L of sterile Microinjection Buffer for 24 hrs. To do so, use the Pierce Slide-A-Lyzer MINI. Load the 50 ul on a column. Close it with the cap and place on the floatation device. Make sure the level of liquid for the construct for microinjection is not below the level of the solution in which you dialyze to avoid diluting out your DNA. Use very gentle stirring. Use a dedicated big beaker, heavily rinsed with water and dd H2O, autoclaved and covered with foil.

12. After the first 24 hrs, exchange the dialysate (Microinjection Buffer) for fresh buffer and dialyze for another 24 hrs.

13. Collect the construct for microinjection and place in a sterile tube.

14. Check OD 260/280 and run a gel with 1 ul of the eluate. The gel should show a single band of the expected size. The Core requires a final concentration of 100-200 ng/ul, a 260/280 ratio between 1.7-2.0 and a final volume of 30-50 ul.

Adapted from protocol provided by Mireia Guerau-de-Arellano, Ph.D.
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