CTAB protocol for extracting DNA from Douglas-fir seed embryos and megagametophytes

The protocol described by Wagner et al. (1987)[1] was downscaled for 1.5 ml microcentrifuge tubes. The embryo/megagametophyte was ground directly in the wash buffer without using spermine or spermidine, then incubated at 65ºC for 15 min. One extraction with phenol/chloroform/isoamyl alcohol (25:24:1) was done after this incubation, 10 µg of RNase A were added, then the tubes were incubated at 37ºC for 1 h. This step was followed by two more extractions with phenol/chlorophorm/isoamyl alcohol (25:24:1). The final extraction was ethanol-precipitated and the pellet was eluted in TE buffer (10:1, pH 8.0).

Step-by-step protocol as described by Aagaard (1997)[2]

1. Soak seeds in dH2O for 2+ hrs. to overnight - can be done dry, but it is more difficult to get a clean separation of the embryo from the megagametophyte.
2. Remove seed coat using a scalpel, dissecting forceps, and dissecting scope.
3. Put the tissue from each embryo/megagametophyte in a 1.5 ml microcentrifuge tube (special grinding tube - Brinkman Eppendorf micro test tube cat.# 22 36 380-8) containing 30ul CTAB wash buffer. Keep tubes and wash buffer on ice until step #6, then work at room temperature.
4. Homogenize the tissue using a pellet pestle and electric drill for about 20 sec/sample.
5. Add 8 volumes (240ul) of wash buffer.
6. Add 1/5 volume (54ul) of 5% sarkosyl. Mix gently by inversion and incubate at room temp. for 3-5 min.
7. Add 1/7 volume (46ul) of 5M NaCl and mix gently by inversion.
8. Add 1/10 volume (37ul) of 8.6% CTAB in 0.7M NaCl and mix gently by inversion.
9. Incubate for 15 minutes in a 65°C waterbath mixing periodically.
10. Extract with one volume (410ul) of phenol:chloroform:isoamyl alcohol (25:24:1). Mix gently by inversion, and spin in a microfuge at maximum speed (13,000 rpm or 14926 g) for 5-10 minutes. Transfer aqueous phase containing DNA (upper part) to a new microfuge tube being careful to avoid whitish interface layer. Save phenol to re-extract for maximum yield.
11. Carefully add 50ul of TE to tube with phenol and interface. Pull off aqueous layer as before and add to the tube in #10. Discard phenol in appropriate container.
12. Add 10ul of RNase (1mg/ml) and incubate in a 37°C waterbath for 30 minutes to 2 hrs.+. Germinated seed - use 20ul of RNase (1mg/ml) and incubate for 1 hour or more.
13. Extract with one volume (410ul) of phenol:chloroform:isoamyl alcohol (25:24:1). Mix gently by inversion and spin in a microfuge at max. speed (13,000 rpm or 14926 g) for 6 minutes. Transfer aqueous phase containing DNA (upper part) to a new microfuge tube being careful to avoid interface layer. Save phenol. Add 50ul TE to tube with phenol. Pull off aqueous layer and add to first tube.
14. REPEAT step #13. Add 20ul of 5M NaCl. This is to adjust for the increase in volume caused by adding 50ul of TE after each Phenol extraction. (1/7 volume of volume change from step 7).
16. Precipitate DNA by adding 2.5 volumes (1ml) of ice cold 95% ethanol and incubate at -20 °C for 30 min. to overnight.

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[1] Wagner DB, Furnier GR, Saghai-Maroof MA, Williams SM, Dancik BP, Allard RW (1987) Chloroplast DNA polymorphisms in Lodgepole and Jack pines and their hybrids. Proc Natl Acad Sci USA 84:2097-2100

[2] Aagaard JE (1997) Genetic diversity and differentiation in Douglas-fir from RAPD markers of nuclear and mitochondrial origin. MS Thesis, Oregon State University, Corvallis, OR, USA

17. Spin at max. speed, at 4° C for 30 min in microfuge. Pour off supernatant being careful not to lose the pellet.
18. Wash the pellet in 1 ml of cold 70% ethanol being careful not to lose the pellet. Dry in SpeedVac for 3-5 minutes.
19. Solubilize pellet in 50ul TE. (for germinated megagametophytes - 25ul TE) Optional - Place in 65° C water bath for 5 min. to dissolve pellet. Clean DNA usually dissolves easily. I prefer to take Fluorometer readings after dissolved pellets sit overnight in the refrigerator.
20. Determine DNA concentration with Fluorometer using a 100 ng/ul standard. Expect 1.5 - 3.0ug DNA/Douglas fir megametophyte.
21. Determine DNA quality - 0.8% TAE agarose gel - 1ul to 5ul of sample depending on DNA concentration.

Solutions

Wash Buffer
0.64g Sorbitol (0.35M)
0.5ml 1M Tris pH8 (50mM)
0.5ml 0.5M EDTA (25mM)
8.36ml d-H2O
10ul 2-mercaptoethanol 0.1% - add just before use!

5% Sarkosyl
5g N-Lauroylsarcosine
d-H2O to 100ml

5M NaCl
146.13g NaCl
d-H2O to 500ml

8.6% CTAB in 0.7M NaCl
80ml d-H2O
8.6g Hexadecyltrimethylammonium Bromide (C-TAB)
heat gently to dissolve
4.09g NaCl
d-H2O to 100ml

10mg/ml RNase A
50mg RNase A
50ul 1M Tris-HCl pH7.5 (10mM)
15ul 5M NaCl (15mM)
d-H20 to 5 ml