PLANT
DNA BARCODE
PROJECT

PCR and Sequencing Protocols - *rbc*L

I. PCR protocol for *rbc*L marker

Note: Phusion® High-Fidelity DNA polymerase was tested on a broad range of plant taxonomic groups and selected as the enzyme with the highest performance for PCR amplification of the chloroplast barcode markers.

rbcL primers

rbcLa-F	ATGTCACCACAAACAGAGACTAAAGC	Levin et al, 2003
rbcLa-R	GTAAAATCAAGTCCACCRCG	Kress & Erickson, 2009

PCR reagents per 10 µL reaction

# of reactions	**1**	**100**
5X HF Buffer (with $MgCl_2$)	2 µL	200 µL
100% DMSO	0.3 µL	30 µL
10 mM dNTPs	0.056 µL	5.6 µL
10 µM Primer Forward	0.1 µL	10 µL
10 µM Primer Reverse	0.1 µL	10 µL
ddH2O	6.32 µL	632 µL
Phusion High Fidelity Fisher Scientific #-530 (5U/ µL)	0.125 µL	12.5 µL
Total	**9 µL**	**900 µL**
DNA template	1 µL per reaction	

Recommendation: taking into account pipette error, aliquot approximately 73 µL of the PCR cocktail in each of the upper 12 wells of the plate. Using a 12-channel pipette, transfer approximately 8.7 µL of the PCR cocktail into each well. Add 1 µL of DNA. Centrifuge the plate before thermocycling.

PCR Thermocycling Program for *rbc*L marker

98°C for 45 seconds;
35 cycles of 98°C for 10 seconds, 55°C for 30 seconds, 72°C for 40 seconds;
final extension 72°C for 10 minutes.

PLANT
DNA BARCODE
PROJECT

II. Sequencing protocol for *rbc*L marker

Dilute PCR product adding 15 µL of water in each well. Centrifuge the plate.

Use the same primers as for PCR.

Sequencing reagents per 10 µL reaction

# of reactions	**1**	**104**
5X Sequencing Buffer	1.875 µL	195 µL
100% DMSO	0	0
10 µM primer	1 µL	104 µL
BigDye	0.250 µL	26 µL
ddH2O	5.875 µL	611 µL
Total	**9 µL**	**936 µL**
Diluted DNA	2 µL per reaction	

Recommendation: taking into account pipette error, aliquot approximately 78 µL of the sequencing mix in each of the upper 12 wells of the plate. With a 12-channeled pipette, transfer approximately 9.5 µL of the PCR cocktail in each well. Add 2 µL of DNA. Centrifuge the plate before thermocycling.

Sequencing Thermocycling Program for *rbc*L marker

94°C for 10 seconds;
35 cycles of 94°C for 20 seconds, 50°C for 20 seconds, 60°C for 4 minutes;
hold at 4°C.

Sequencing Cleanup

- Add Sephadex powder to the Acroprep 96 filter plate. The standard amount of powder is measured by a column loader.
- Add 300 µL of dH2O. Let Sephadex hydrate for 2 hours at room temperature or overnight at 4°C.
- Assemble the Sephadex plate onto collection plate and centrifuge at 2100 rpm for 5 minutes.
- Immediately proceed to load the sequencing product onto the Sephadex columns to avoid drying. Use a fresh plate as a collecting plate.

- Centrifuge at 2100 rpm for 5 minutes.
- Dry the cleanup product at 88°C for 20 minutes, then cover the plate with a rubber lid and place in the freezer at -20°C until it is placed in ABI capillary sequencer.
- * The front panel of the plate should be labeled by the standard order number generated by Smithsonian GeneSifter (https://smithsonian.genesifter.net/login).

References

Kress WJ, Erickson DL, Jones FA, Swenson NG, Perez R, et al. (2009) Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. Proceedings of the National Academy of Sciences 106: 18621–18626.

Levin RA, Wagner WL, Hoch PC, et al. (2003) Family-Level Relationships of Onagraceae Based on Chloroplast rbcL and ndhF Data. American Journal of Botany, vol 90:107-115 (modified from Soltis P et al. (1992) Proceedings of National Academy of Sciences USA, 89: 449-451.