PCR and Sequencing Protocols - \textit{rbcL}

I. PCR protocol for \textit{rbcL} marker

\textit{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.

\textit{rbcL} primers

\begin{tabular}{|l|l|l|}
\hline
\textit{rbcLa-F} & ATGTCACCACAACAGAGACTAAAGC & Levin et al, 2003 \\
\textit{rbcLa-R} & GTAAATCAAGTCACCRCG & Kress & Erickson, 2009 \\
\hline
\end{tabular}

PCR reagents per 10 μL reaction

\begin{tabular}{|l|l|l|}
\hline
\textbf{# of reactions} & \textbf{1} & \textbf{100} \\
\hline
5X HF Buffer (with MgCl\textsubscript{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 \\
\textbf{Total} & \textbf{9 μL} & \textbf{900 μL} \\
DNA template & 1 μL per reaction & \\
\hline
\end{tabular}

\textit{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.

\textbf{PCR Thermocycling Program for \textit{rbcL} 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.
II. Sequencing protocol for *rbcl* 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

<table>
<thead>
<tr>
<th># of reactions</th>
<th>1</th>
<th>104</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Sequencing Buffer</td>
<td>1.875 μL</td>
<td>195 μL</td>
</tr>
<tr>
<td>100% DMSO</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 μM primer</td>
<td>1 μL</td>
<td>104 μL</td>
</tr>
<tr>
<td>BigDye</td>
<td>0.250 μL</td>
<td>26 μL</td>
</tr>
<tr>
<td>ddH2O</td>
<td>5.875 μL</td>
<td>611 μL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>9 μL</strong></td>
<td><strong>936 μL</strong></td>
</tr>
<tr>
<td>Diluted DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 μL per reaction</td>
<td></td>
</tr>
</tbody>
</table>

*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 *rbcl* 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
