the course mesh back into the omnimixer and mix with the remaining 100 mL of nuclei extraction buffer.

7. Add NP40 to 0.6%, and filter this slurry through the meshes. Combine this with the first filtered slurry.
8. Transfer the filtered slurry to 250-mL centrifuge bottles and centrifuge for 10 min at 4000*g* in a Sorvall GSA rotor. If the detergent has worked as expected, a dark-green mass of disrupted chloroplast fragments should float to the top of the bottles. The pellet will contain the nuclei and will be a grayish green. Using a vacuum, remove the top layer carefully, then carefully aspirate the buffer from the pellet. Wipe the sides of the bottles with kimwipes to remove the rest of the dark-green supernatant.
9. Gently resuspend the pellets in 95% Percoll stock solution and distribute to four 30 mL silanized Corex tubes (we use silanized tubes, because the mixtures tend to stick to the surface of unsilanized glass). Bring each tube to ~15 mL with the 95% Percoll stock, gently mix, then gently pipet a 10-mL layer of mannitol buffer over the resuspended Percoll pellets.
10. Centrifuge at 4°C for 10 min at 4000*g* in a Sorvall HB-4 swinging-bucket rotor.
11. Collect nuclei from the mannitol buffer/95% Percoll interface in each tube. The material at this interface should be light-green and very viscous. Use a bent-tip 15-gage, or larger, diameter needle, or a similar wide-bore device, attached to a syringe, to collect the viscous band.
12. Distribute the nuclei to two fresh 30-mL glass tubes, and dilute the nuclei in each tube to 15 mL with mannitol buffer. After dilution, the concentration of Percoll should be less than 50%.
13. Underlayer 10 mL of the 75% Percoll stock solution to make a cushion on the bottom of each tube.
14. Centrifuge for 5 min at 4000*g* in the HB-4 rotor.
15. Collect the nuclei from the interface and the top half of the 75% Percoll layer and pool them in one fresh 30-mL tube (*see* **Subheading 4.4., Note 2**).
16. Dilute the nuclei to 25 mL with Mannitol buffer, and centrifuge for 5 min at 1000*g* in the HB-4 rotor. Aspirate the supernatant from the nuclear pellet. At this stage, the very loose pellet will be about 1–2 mL. Shake the pellet gently to resuspend the nuclei.
17. Transfer the nuclei from the pellet to 1.5-mL Eppendorf centrifuge tubes in 500-µL batches, using a wide-bore instrument (e.g., a Pipetteman P-1000 with a cutoff tip). If the pellet is too viscous to pipet, adding a small amount (~1–2 mL) of mannitol buffer will facilitate the transfer.
18. To recover the polymerase from the nuclei, add 500 µL of nuclei to 1.5-mL Eppendorf centrifuge tubes, and an equal volume of polymerase elution buffer to each tube. Then, add 50 U of RNasin (Promega) to each tube. Rock the tubes gently for 30 min at 4°C.
19. Centrifuge the tubes for 30 min at 16,000*g* in an Eppendorf microcentrifuge at 4°C.
20. Collect the supernatants from the tubes, pool, and mix the supernatants and store as 200-µL aliquots. Freeze the polymerase extract in liquid nitrogen and store at