96-well plate CTAB extraction

*It should be noted that this is a modified version of a protocol provided by Sandra Boles, formerly of the Shaw Laboratory, Duke University Department of Biology. We extend our sincere thanks to Sandy and to Jon Shaw for both the protocol and considerable guidance as we adapted it for our taxa.

*All photos were taken by Patrick Alexander (New Mexico State University).

*This protocol was written for the simultaneous extraction of (2) 96-well plates (192 samples total). Working with two plates at a time allows for easy centrifuge balancing in later steps, although smaller numbers of extractions could be performed as long as an even number of strip tubes are used.

*Before starting you will need to purchase a set of 1.1ml "mini-tubes." These are sold as 8-tube strips in a convenient 96-well rack (12 strips per rack), are manufactured by Axygen or Netpune, and available through Fisher and VWR. Be sure to purchase the appropriate caps for the tubes as well. Drill several small diameter holes in a few of the racks to aid in the nitrogen/incubation steps below.

A. Grinding and extracting with CTAB buffer

1. Add 2 (5/32" diam.) stainless steel ball bearings (Royal Steel Ball, Sterling IL) to each tube.

2. Label the bottom ("H" row) row of tubes H1-H12 and the top ("A" row) row of tubes A1-A12.

3. Add a small amount of tissue from a target specimen into each tube. The amount will vary by species, but for *Boechera* approx. 10 mg of tissue yields approx. 300 ng/µl of DNA if the final elution is in 100 µl TE. Be sure to keep track of specimen identity/tube number.

4. Cap the tubes with the small strip cap tabs towards the top (A row) to make removal easier. Label the tabs A1-A12. Ensure the tubes are in the 96-well racks with holes drilled in the bottoms, which will enable the liquid nitrogen and hot water to infiltrate in later steps.

5. Set a water bath to 65°C and ensure that the volume is appropriate for a 96-well rack. It should contain sufficient water to submerge the bottom half of the tubes, but not so much as to submerge the entire tube.

6. Prepare fresh CTAB/BME isolation buffer (0.2% β-mercaptoethanol) by adding β-mercaptoethanol to 2X stock CTAB buffer in a flask. Keep warm on a hot plate.

7. Pour a small amount of liquid nitrogen into a styrofoam cooler.

8. Freeze the tissue by placing the 96-well racks (with holes in the bottoms) loaded with the 12 firmly capped 8-tube strips in the liquid nitrogen. Metal centrifuge carriers are helpful for lowering and transferring the racks into and out of the liquid nitrogen.

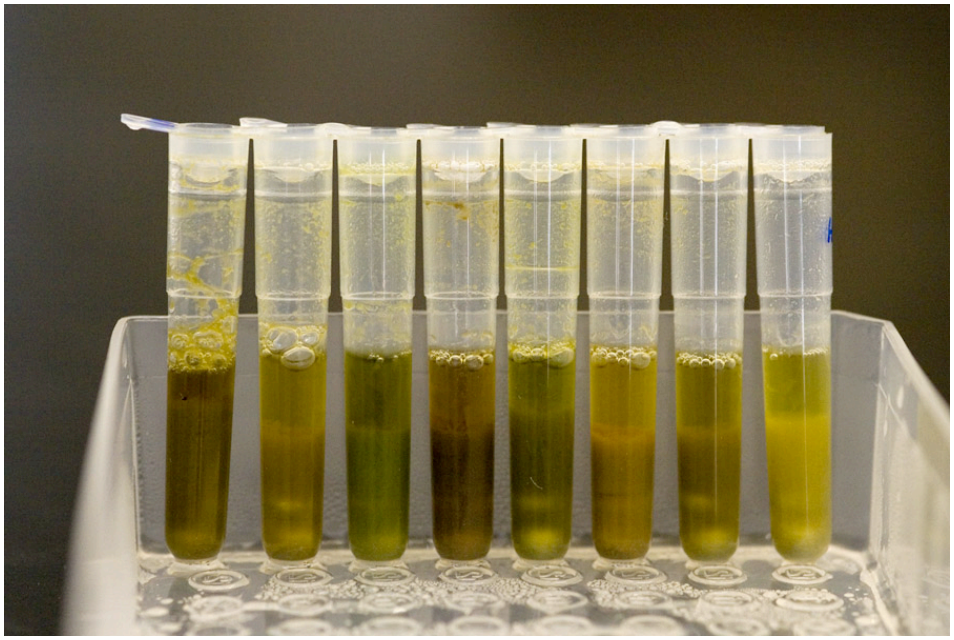
9. After ca. 10 seconds remove the frozen racks and immediately transfer to a Genogrinder or similar tissue disrupter. We used a 2000 GenoGrinder (SPEX, Metuchen, NJ), which shakes clamped plates (2 at a time) vertically at high speeds. An alternative is the reciprocating saw approach published by Alexander et al. (2007) Molecular Ecology Notes 7: 5-9.

10. Ensure that the two racks are tightly clamped. If using a GenoGrinder it is critical that the clamp presses directly down on the strip tube caps themselves (remove the 96-well rack lid). Shake at the "1×/ 500 speed" (1500 strokes/minute) setting for 1 minute. Note that if cracked tubes are observed (you'll notice them in later liquid steps) you can opt for lower speeds and/or a shorter shake time. Open the Genogrinder and remove your racks.

11. Gently remove strip caps (on a paper towel), being sure to note cap identity/orientation and taking care not to spread powdered tissue. Flicking each 8-strip tube a couple times will help knock excess powdered tissue to the bottom of the tubes. Removing the caps will take some time, as the tubes will still be somewhat hardened from the liquid nitrogen step.

12. Add 400 µl prepared, warm CTAB buffer to each sample with a 1200 µl multi-channel. The CTAB can be poured into a plastic reservoir for pipetting with the multi-channel. This is best done on the open lab bench, as the powdered tissue can be drawn up by the suction of a fume hood.

13. Carefully re-cap the tubes, taking care to add the caps to the corresponding strip tubes in the correct orientation. Vortex each 8-tube strip vigorously to mix the powdered tissue with the CTAB. Try to get as much of the tissue in solution, but don't worry if a small pellet in the bottom of the tube won't dislodge. At this point one of the 8-tube strips will look something like this:



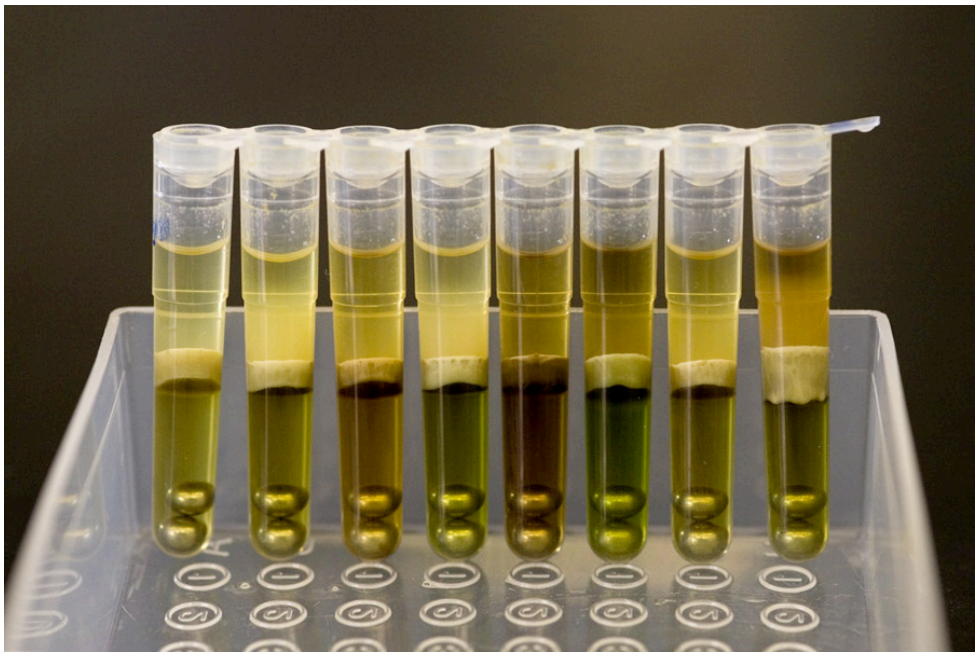
14. Return all 8-tube strips to their racks (again the ones with holes), add the rack lids, and incubate in the 65°C water bath for 1 hour. Be sure to place a weight of some kind (a spare aluminum dry block works great) on the rack lid to prevent tube opening during the incubation.

15. Remove racks (keep weight on!) and allow to drain and cool for approx. 15 minutes in a tub or tray.

B. Removal of proteins with chloroform:isoamyl alcohol

16. In a hood, pour the required volume of (24:1) chloroform:isoamyl alcohol into a glass dish for pipetting (the mixture will dissolve a plastic reservoir). Add an equal volume (400 µl) of the (24:1) chloroform:IAA to the plant extract with a 1200 µl multichannel pipette. Cap tubes securely and add a folded paper towel to the inside of the rack lid to fill the small gap between the tube caps and the lid. Mix by inverting each rack (30-50× by hand) to produce an emulsion.

17. Centrifuge the two (balanced!) racks with lids for 5 minutes at 3700 rpm in a plate centrifuge. This can be done at a higher rpm, but 3700 is sufficient in our experience. After centrifugation one of the 8-tube strips will look something like this:

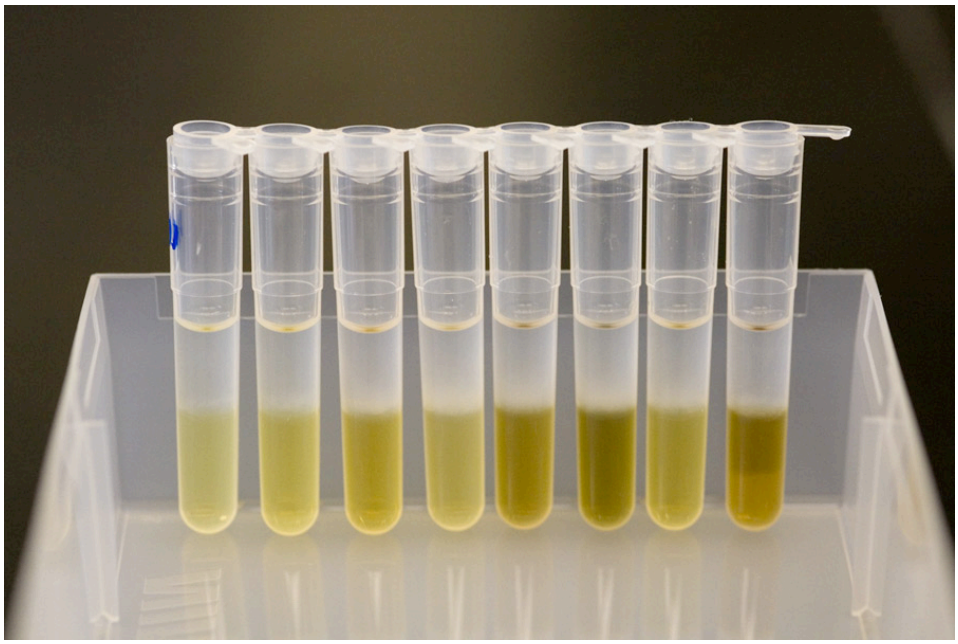


18. Remove aqueous (top) phase to new, labeled 8-strip tubes using a 1200 µl multi-channel. Removing the top phase is difficult with a large volume multi-channel but can be done if you set the volume for approx. 300 µl, angle the tubes, and go slowly. Take note of the general volume of transferred aqueous layer for step #20 below. Avoid disturbing or pipetting any of the white interface, although transferring a small amount of this layer has not appeared to have serious consequences for our extractions.

19. Dispose of the tubes, ball bearings, plant extract, and chloroform/IAA waste according to your university regulations.

C. Precipitation, washing, drying, and re-suspension of DNA

20. Add an equal volume of cold isopropanol to the aqueous phase, after which you should see a white precipitate at the boundary. At this point one of the 8-tube strips will look something like this:



21. Cap tubes securely, rack them, add a folded paper towel to the inside of the rack lid to fill the small gap between the tube caps and the lid, and mix gently (ca. 10× by hand) by inverting each rack.

22. Precipitate at -20°C overnight. Note that one could likely precipitate for far less time.

23. Centrifuge the (balanced!) racks for 20 minutes at 3700 rpm in a plate centrifuge. When the run ends a white, off-white, or even darkly colored pellet will potentially be visible at the bottom of many tubes. Although no pellet is visible in certain taxa, considerable DNA was nevertheless recovered.

24. Remove caps. Working with one 8-tube strip at a time, pour off the isopropanol (a deep glass dish or petri dish works well) and then remove the isopropanol remaining in the tube by dragging the tube strip along a clean paper towel. Be sure to drag quickly and in a straight line to avoid cross-contaminating tubes with isopropanol from adjacent tubes. Carefully monitor any visible pellets to ensure they are not being poured off. Note that if you wait too long after the centrifuge run ends many of the pellets detach from the tubes making it difficult to pour.

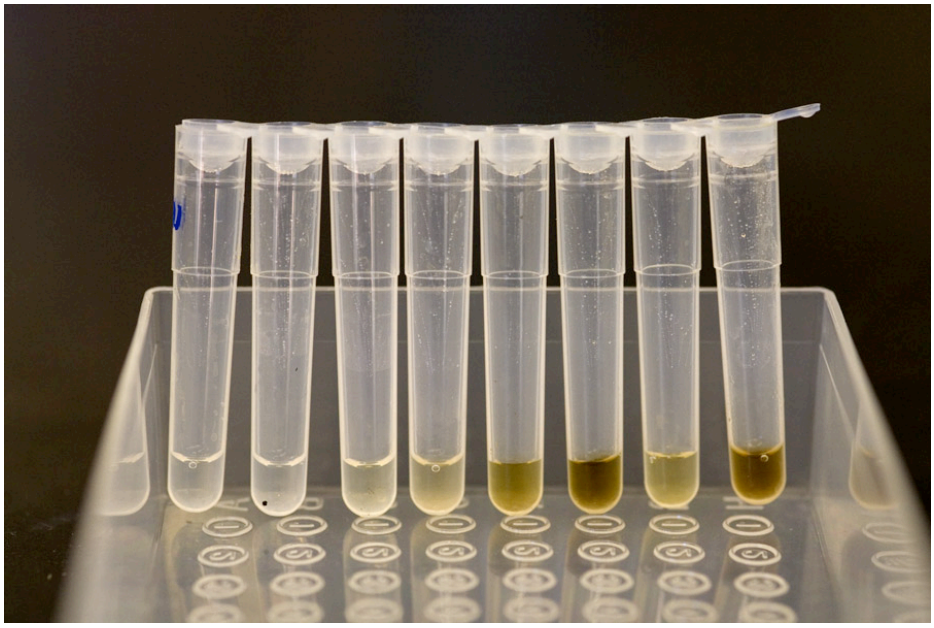
25. Wash pellets by adding 500 µl 70% ethanol, securely capping tubes, and gently vortexing each 8-tube strip individually. With sufficient vortexing most pellets will release from the tube bottoms and rotate in the ethanol.

26. Cap tubes securely, rack them, and spin the two (balanced!) racks 10 minutes at 3700 rpm in a plate centrifuge to secure pellets to tube bottoms.

27. Remove caps and pour off the ethanol as you did the isopropanol in step #24.

28. Do not re-cap the tubes. Air-dry pellets by covering the racked, uncapped tube strips with a clean paper towel, leaving them out overnight on a benchtop at room temperature.

29. Add 50-100 µl TE, re-cap tubes, and incubate racked tubes for 1-2 hours at 37°C (vortexing periodically) to assist with re-suspension. Following re-suspension one of the 8-tube strips will look something like this:



30. Note that re-suspended DNA extracts may exhibit a range of colors from clear to black depending on the age and quality of the extracted tissue. Proceed with your preferred DNA quantification methodology, DNA dilutions, and downstream applications. GOOD LUCK!