**This protocol was created by Katherine Moccia, Univ. of TN as adapted from Shade et al. 2013. Josh H. tweaked this a tiny bit too.**

*Note: one can stop at any step of this protocol, just stick samples back in the freezer. Label them so folks know whose they are and what they are. Make sure it is clear at which point in the protocol you stopped. Let them thaw out before continuing with the protocol.*

1. Store samples on ice for collection and transportation.

Make sure tubes are labelled prior to putting plant material in them. You can use regular microcentrifuge tubes, but they should be sterile (autoclave them if in doubt).

3. Weigh samples into tubes. Need .05 grams of fresh, wet plant material. Can be approximate, but try to get over 0.04 g. Do not bother writing down weights of tubes at this step. Avoid stems, flowers, seeds, and ugly ass leaves. Try to get healthy leaves if possible. Consider counting the number of leaves/leaflets sampled. For alfalfa, we will count leaves.

Tips: use scissors and forceps to remove leaves from stems. Work over a bin/table of some sort that you can sterilize with dilute bleach, that way if you drop a leaf it isn’t a big deal. Flame forceps/scissors between samples if possible.

4. Add 500 uL of 1x PBS, 0.15% Tween 20 to each tube

5. Shake for 20 minutes at 4C on a rotor in a polysteryene foam container filled with ice.

Tips: use the shaker table in BC 320 or ecolab. Shaking rate should be around as high as the thing goes, since this is the step we are using to try and dislodge epiphytes. The shaker table is sort of cryptic to figure out, but persevere and you can do it! Details can be added here if anyone figures out an easy way to explain it. I find it easiest to just play with the thing and get it going. Note this is sort of loud.

6. Sonicate leaves for 5 minutes in a water bath. Use warm water. Swap water between batches of samples. Make sure the tubes aren’t cracked and leaky before you do this. If they are, then put the sample in a new tube that is labelled.

7. Transfer the supernatant (this is the epiphyte sample; use a pipette) to a new sterile, weighed, tube (should be a plain microcentrifuge tube as safelocks are pricey) make sure it is labelled and says “ep” for epiphyte. Record weights of these tubes before and after addition of supernatant.

Tip: it can be nice to use different colored tubes for epiphytes than endophytes.

8. Store frozen if it will be awhile till extraction. Otherwise, go straight to extraction. Make sure it is clear where these samples are being stored in your notebook. If the LIMS allows, samples can be input into the system at this point. We may need to keep them in the Buerkle lab if space is an issue.

9. With the remaining plant material (endophyte sample) poke holes in the top of each tube (using a hot forcep, or some other hot metal poker. JH bought a poker for this), freeze in -80 (flash freezing is not needed, the -80 works quick enough).

10. Freeze dry overnight or until properly dried. The goal here is to get dry samples that are easier to grind using the tissue lyser. Tip: use the freeze drier in the ag building because the one in the biogeochem lab is weak. Contact Paul to get access to the ag building and freeze drier.

Tips: to use the lyophilizer make sure vacuum is down and temp is down. There are guidelines for settings on the instrument. Make note of the settings you used. Make sure the glassware is attached to the lyophilizer well bc it is expensive and we don’t want it to fall off. Don’t forget to open the valves that go to each glassware to turn on the vacuum.

10. Once dried, put in a new labelled, weighed, safelock tube. Lyse via bead mill. Make sure samples are totally powderized. This is the most important step for getting good DNA yield. Dump out bead and weigh tube. This weight will be how much mass we extract DNA from.

11. Store the rest of the dried sample at -80°C.

