DNA isolation from small amounts of plant tissue

Background. This procedure was developed in 1984 as an alternative to the lengthy, expensive, and low-yield cesium chloride-ethidium bromide ultracentrifugation procedure that required the isolation of nuclei, and which produced only degraded DNA from soybean leaves in our hands. In looking for a method that used small amounts of tissue, we found a paper by Saghai-Maroof et al. (PNAS 81:8014-8019, 1984) that was in turn modified from a procedure developed in the laboratory of Arnold J. Bendich (U. of Washington), which used the strong detergent CTAB. However, the Saghai-Maroof et al. procedure used lyophilized tissue, and there was no lyophilizer in the lab of Roger Beachy at Washington University in St. Louis, where we were working (JJD was a postdoc, JLD was a technician). We tried the Saghai-Maroof procedure exactly as described, but this failed with soybean leaves. We therefore modified the procedure by doubling the concentration of components of Saghai-Maroof et al.'s extraction buffer to compensate for the greater water content of fresh tissue. This very simple modification worked beautifully.

We did not publish the procedure because it was such a simple modification of existing procedures, until asked to do so by the editor of the *Phytochemical Bulletin*. This was at the time that DNA variation was just beginning to be used as a source of characters for plant systematics studies, and interest was high; we were sending the procedure to many colleagues, so this seemed reasonable. *Phytochemical Bulletin* was the publication of the Phytochemical Section of the Botanical Society of America, and in 1987 it was a set of typewritten sheets stapled together. It does not appear to have been accessioned in any libraries, so it is impossible to find. In 1987, JJD and EE Dickson tested the procedure on herbarium specimens and preserved tissues, and published the results in the plant systematics journal, *Taxon*, but this paper is rarely cited even though it gives the same procedure.

Because of the inaccessibility of the original protocol, and given the ever-growing need for plant DNA minipreps, we were asked by the editor of *Focus* in 1990 to publish the protocol there, and did so. Unfortunately, *Focus* was also not a "real" scientific journal, but instead was the trade publication of GIBCO-BRL, Inc., so it cannot be found in libraries, either. Ironically, we discovered in 1990, while on a seminar trip to Texas A&M University, that Dr. Brian Taylor of that university had published an identical procedure, in *Focus*, in 1982 (Taylor & Powell, *Focus* 4:4-6), of which we were completely unaware! He had developed it directly from a Bendich procedure he had learned while at the University of Washington.

In 1990 we taught the technique to a large group of people at a NATO workshop on "molecular taxonomy" held in Norwich, England. The proceedings were published, so that the procedure has now appeared four different times:

Doyle, J. J. and E. E. Dickson. 1987. Preservation of plant samples for DNA restriction endonuclease analysis. Taxon 36:715-722.

Doyle, J. J. and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19: 11-15.

Doyle, J. J. and J. L. Doyle. 1990. A rapid total DNA preparation procedure for fresh plant tissue. Focus 12:13-15.

Doyle, J. J. 1991. DNA protocols for plants. pp. 283-293 in: G. Hewitt, A. W. B. Johnson, and J. P. W. Young (eds.), *Molecular Techniques in Taxonomy*. NATO ASI Series H, Cell Biology Vol. 57.

Doyle & Doyle CTAB Procedure:

1. Preheat 5-7.5 ml of CTAB isolation buffer (2% hexadecyltrimethylammonium bromide [CTAB: Sigma H-5882], 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0) in a 30 ml glass centrifuge tube to 60^{o}C in a water bath.

2. Grind 0.5-1.0 g fresh leaf tissue in in 60^{o}C CTAB isolation buffer in a preheated mortar.

3. Incubate sample at 60^{o}C for 30 (15-60) minutes with optional occasional gentle swirling.

4. Extract once with chloroform-isoamyl alcohol (24:1), mixing gently but thoroughly.

   This produces two phases, an upper aqueous phase which contains the DNA, and a lower chloroform phase that contains some degraded proteins, lipids, and many secondary compounds. The interface between these two phases contains most of the "junk"--cell debris, many degraded proteins, etc.

5. Spin in clinical centrifuge (swinging bucket rotor) at room temperature to concentrate phases. We use setting 7 on our IEC clinical (around 6,000 x g) for 10 min.

   This is mainly to get rid of the junk that is suspended in the aqueous phase.

   Generally the aqueous phase will be clear, though often colored, following centrifugation, but this is not always the case.

6. Remove aqueous phase with wide bore pipet, transfer to clean glass centrifuge tube, add 2/3 volumes cold isopropanol, and mix gently to precipitate nucleic acids.

   A wide bore pipet is used because DNA in solution is a long, skinny molecule that is easily broken (sheared) when it passes through a narrow opening. Gentleness also improves the quality (length) of DNA.

   In some cases this stage yields large strands of nucleic acids that can be spooled out with a glass hook for subsequent preparation. In most cases, this is not the case, however, and the sample is either flocculent, merely cloudy-looking, or, in some instances, clear. If no evidence of

precipitation is observed at this stage, the sample may be left at room temperature for several hours to overnight. This is one convenient stopping place, in fact, when many samples are to be prepped. In nearly all cases, there is evidence of precipitation after the sample has been allowed to settle out in this manner.

7. If possible, spool out nucleic acids with a glass hook and transfer to 10-20 ml of wash buffer (76% EtOH, 10 mM ammonium acetate).

a. Preferred alternative: Spin in clinical centrifuge (e.g. setting 3 on IEC) for 1-2 min. Gently pour off as much of the supernatant as possible without losing the precipitate, which will be a diffuse and very loose pellet. Add wash buffer directly to pellet and swirl gently to resuspend nucleic acids.

b. Last resort: Longer spins at higher speeds may be unavoidable if no precipitate is seen at all. This will result, generally, in a hard pellet (or, with small amounts, a film on the bottom of the tube) that does not wash well and may contain more impurities. Such pellets are difficult to wash, and in some cases we tear them with a glass rod to promote washing at which point they often appear flaky.

Nucleic acids generally become much whiter when washed, though some color may still remain.

8. Spin down (or spool out) nucleic acids (setting 7 IEC, 10 min) after a minimum of 20 min of washing. The wash step is another convenient stopping point, as samples can be left at room temperature in wash buffer for at least two days without noticeable problems.

9. Pour off supernatant carefully (some pellets are still loose even after this longer spin) and allow to air dry briefly at room temperature.

10. Resuspend nucleic acid pellet in 1 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).

Although we commonly continue through additional purification steps, DNA obtained at this point is generally suitable for restriction digestion and amplification, so we'll stop here.

If DNA is to be used at this stage, pellets should be more thoroughly dried than indicated above.

Gel electrophoresis of nucleic acids at this step often reveals the presence of visible bands of ribosomal RNAs as well as high molecular weight DNA.

11. Add RNAase A to a final concentration of 10 *u*g/ml and incubate 30 min at 37^{O}C.

12. Dilute sample with 2 volumes of distilled water or TE, add ammonium acetate (7.5 M stock, pH 7.7) to a final concentration of 2.5 M, mix, add 2.5 volumes of cold EtOH, and gently mix to precipitate DNA.

    > DNA at this stage usually appears cleaner than in the previous precipitation. Dilution with water or TE is helpful, as we have found that precipitation from 1 ml total volume often produces a gelatinous precipitate that is difficult to spin down and dry adequately.

13. Spin down DNA at high speed (10,000 x g for 10 min in refrigerated centrifuge, or setting 7 in clinical for 10 min).

14. Air dry sample and resuspend in appropriate amount of TE.