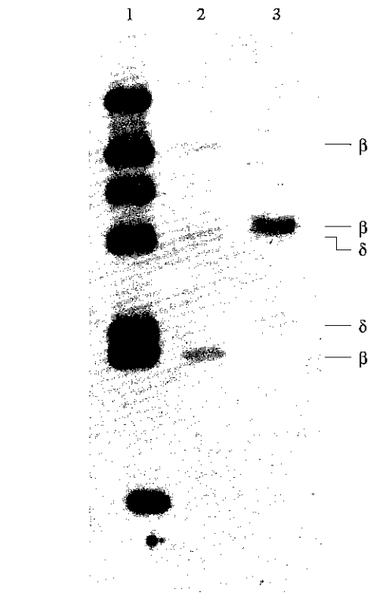

FIGURE 5. Southern blot of human genomic DNA. DNA was digested with restriction endonuclease, electrophoresed in a 1% agarose gel, and transferred to nylon membranes. Filters were hybridized to a biotin-labeled plasmid containing a 4.4-kb segment of the human β-globin gene. The samples are: biotinylated *Hind* III lambda DNA marker fragments (lane 1); 2 µg human diploid fibroblast (MRL-5) DNA, digested with *Bam*H I (lane 2); and 2 µg human diploid fibroblast DNA, digested with *Pst* I (lane 3). The film was exposed for 5 min.

The kinetics of light output are different from the familiar ^{32}P detection. When selecting exposure times and calculating desired reexposure times it is important to remember that the light intensity increases for several hours before stabilizing. This concern can be avoided by incubating the membranes with detection reagent for 3 h before exposing film. The high stability of light production between 3 and 21 h is a clear advantage of the dioxetane system over the enhanced luminol system. Multiple exposures can be easily obtained from a single membrane. This allows multiple exposures to be performed at constant light output to fully optimize photographic exposure.

The dioxetane system was able to detect less than one picogram of target DNA. This is more sensitive than the enhanced luminol system and than NBT/BCIP substrates. However, the full benefits of the sensitivity of the dioxetane substrate have not yet been realized. The film exposures used here are relatively brief, limited to the time in which no background is visible. It is likely that the sensitivity of the membrane-based assay can be increased by reducing nonspecific signal.

In summary, the dioxetane system has a number of attractive features. The film exposures are short, allowing results to be obtained in one day. The results are virtually identical to standard autoradiograms. Unlike the enhanced luminol system, this system accommodates multiple exposures of the same experiment over extended periods of time. Sensitivity is in the subpicogram range, making this system useful for a wide range of applications. The enhanced dioxetane system is available from BRL as the PhotoGene™ Nucleic Acid Detection System.

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* Manufactured under license from Lumigen, Inc., Detroit, MI

ISOLATION OF PLANT DNA FROM FRESH TISSUE

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The isolation of high molecular weight DNA that is suitable for digestion with restriction endonucleases can be a serious stumbling block to progress in molecular studies of many types. Plants are particularly notorious for their intractability with many isolation procedures. Furthermore, a procedure that works with one plant group will often fail miserably with others, an outcome that is not unexpected given the diversity of plants and their secondary compounds.

Older methods for DNA isolation from plants require large amounts of tissue due to low yields, clearly a drawback when DNA must be isolated from numerous small individuals. Furthermore, methods that rely on CsCl gradients are time consuming and expensive. Fortunately, a diversity of less expensive protocols have been developed that are characterized by high yields of DNA from small amounts of tissue. Several of these (1-4) use hexadecyltrimethylammonium bromide (CTAB). Here we describe our version of a procedure for isolation of DNA from fresh plant tissue using CTAB (2), which is a modification of the method of Saghai-Maroof *et al.* (4) with lyophilized leaves.

This CTAB method for fresh tissue has been used successfully in our laboratory on a wide taxonomic sampling of plant families, including both monocots (*e.g.*, palms, grasses, sedges, orchids) and dicots (*e.g.*, walnuts, hickories, oaks, beeches, legumes, apples and relatives, saxifrages, lobelias, brassicas, portulacas), as well as conifers and ferns. The method also has been used successfully with recently dried (up to 2 years), pressed leaves (3) and outside the plant kingdom with insects (5).

PROTOCOL

1. Preheat 5 to 7.5 ml of CTAB isolation buffer [2% (w/v) CTAB (Sigma), 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, (pH 8.0)] in a 30-ml glass centrifuge tube to 60°C in a water bath.
2. Grind 0.5 to 1.0 g of fresh, leaf tissue to a powder in liquid nitrogen in a chilled mortar and pestle.
3. Scrape the powder directly into preheated buffer and swirl gently to mix.

Comment: Alternatively, fresh tissue may be ground in CTAB isolation buffer at 60°C in a preheated mortar.

4. Incubate the sample at 60°C for 30 min with optional occasional gentle swirling.
5. Extract once with chloroform-isoamyl alcohol (24:1; v:v), mixing gently but thoroughly.
6. Centrifuge (1600 x *g*) in a clinical centrifuge (swinging bucket rotor) at room temperature to the concentrate phases.

Comment: Generally the aqueous phase will be clear following centrifugation, but this is not always the case.

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

Comment: In some cases, this stage yields large strands of nucleic acids that can be spooled with a glass hook. Generally, the sample is either flocculent or cloudy. 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 being prepared. In nearly all cases, there is evidence of precipitation after the sample has been allowed to settle out in this manner.

8. Recover the nucleic acid by one of the following options:
   a. If strands of DNA are visible, spool the nucleic acids with a glass hook and transfer to 10 to 20 ml of wash buffer [76% (v/v) ethanol, 10 mM ammonium acetate].
   b. If the DNA appears flocculent, centrifuge at (500 x *g*) for 1 to 2 min. Gently pour off as much of the supernate as possible without losing the precipitate, which will be a diffuse and very loose pellet. Add wash buffer directly to the pellet and swirl gently to resuspend the nucleic acids.

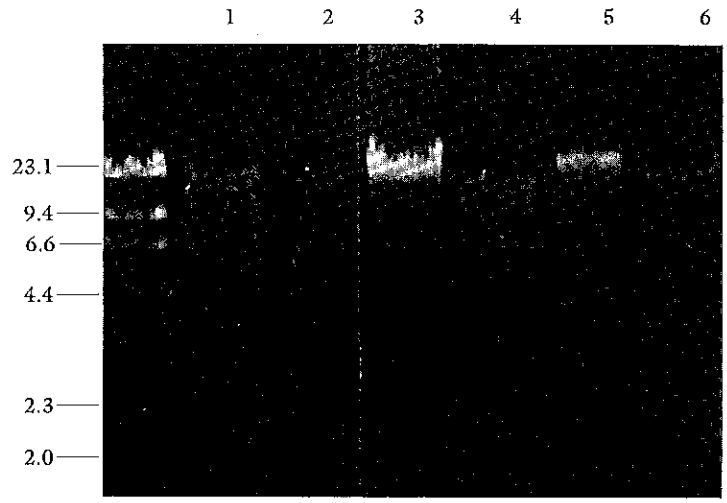


FIGURE 1. DNA isolated from plants by the CTAB procedure. Samples were subjected to electrophoresis on a 0.7% (w/v) agarose gel in Tris-borate EDTA buffer. Lanes: 1, *Alnus* (alder); 2, *Bolusafra* (a legume); 3, *Sphenostylis* (a legume); 4, *Claytonia* (spring beauty); 5, *Salacca* (a palm); and 6, undigested bacteriophage λ DNA. Molecular weight standard is λ DNA digested with *Hind* III. DNA shown in lanes 3 and 5 was isolated using the liquid nitrogen procedure; the remaining samples were isolated without liquid nitrogen.

c. If the precipitate is not obvious, centrifuge the solution at higher speeds. This will generally result in a hard pellet or, with small amounts, a film on the bottom of the tube that may contain more impurities. Such pellets are difficult to wash, and may require stirring with a glass rod to promote washing.

Comment: The nucleic acids generally become much whiter when washed, though some color may still remain.

9. Centrifuge (1600 x *g* for 10 min) or spool the nucleic acids after a minimum of 20 min of washing.

Comment: The wash step is another convenient stopping point. Samples can be left at room temperature in wash buffer for at least two days without noticeable problems.

10. Pour off the supernate carefully and allow the pellet to air dry briefly at room temperature.
11. Resuspend the pellet in 1 ml TE [10 mM Tris-HCl (pH 7.4), 1 mM EDTA].

Comment: Gel electrophoresis of the nucleic acids at this step often reveals the presence of visible bands of rRNAs, as well as high molecular weight DNA. Although we commonly continue with steps 12 through 15, the DNA at this stage is generally suitable for restriction digestion. If the DNA is used at this stage, the pellets should be dried more thoroughly than indicated in step 10.

12. Add RNase A to a final concentration of 10 μg/ml and incubate 30 min at 37°C.
13. Dilute the sample with 2 volumes of distilled water or TE and add 7.5 M ammonium acetate (pH 7.7) to a final concentration of 2.5 M and 2.5 volumes of cold ethanol. Gently mix to precipitate the DNA.

Comment: 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 a 1 ml total volume often produces a gelatinous precipitate that is difficult to pellet and dry adequately.

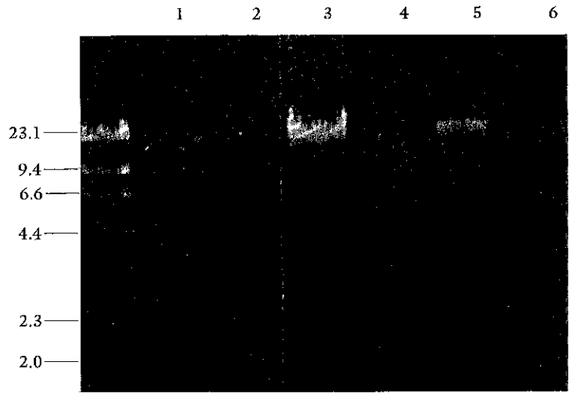
14. Centrifuge the DNA 10,000 x *g* for 10 min in a refrigerated centrifuge.
15. Air dry the sample and resuspend in an appropriate amount of TE.

RESULTS AND DISCUSSION

This procedure yields total DNA, both nuclear and chloroplast sequences (data not shown). The DNA is generally high molecular weight (figure 1), although often some low molecular weight fragments are observed. The difference in DNA quality between samples shown is representative of most plant groups. The liquid nitrogen procedure consistently yields DNA of higher average molecular weight than does the method in which fresh tissue is ground directly in buffer. However, for most applications, such as screening large numbers of individuals, we routinely use fresh-ground samples.

Yields using these methods often approach 1 mg/g fresh tissue, although this is strongly dependent on both the age and quality of the tissue and on the species used. Quantification by absorbance at 260 nm generally gives unreliable results, presumably due to interference of residual CTAB in the samples.

Although the basic procedure described here has been used with many different plant groups, modifications may improve the quality or yield of DNA in some plant groups. For example, in plants with high concentrations of phenolic compounds, such as oaks and walnuts, 1% (w/v) polyvinylpyrollidine (PVP-40) has been added to the isolation buffer with successful results (unpublished observations). For plants containing high polysaccharide levels and/or glutinous sap, which often yield very viscous grindates (*e.g.*, *Onagraceae*, bromeliads),



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In some cases, DNA obtained by this procedure is further purified by one or two ultracentrifugation steps in CsCl. This modification combines the advantages of the high yields routinely achieved by the CTAB isolation method with the presumably greater purity of CsCl methods. In many protocols, however, we use this CTAB procedure as a substitute for CsCl gradients. For example, chloroplast DNA isolations may be performed more rapidly and with no apparent detrimental effect by following published protocols for chloroplast DNA isolation (7, 8) up to the point of obtaining a fraction enriched for chloroplasts or DNA. An equal volume of preheated CTAB isolation buffer is then added, and the protocol given here is followed.

The method described here is readily modified for very small amounts of fresh tissue. We have performed population surveys with DNA from over 200 individual plants, often using 0.01 to 0.1 of fresh tissue per plant (9). In such cases, the liquid nitrogen is omitted. Grinding, incubation in isolation buffer, chloroform-isoamyl alcohol extraction, and centrifugation may all be performed in a single microcentrifuge tube. The DNA, in this case, is used after the first precipitation. DNA also may be isolated from dried seeds; however, the large amounts of starch and protein in seeds often require additional extraction with organic solvents and further purification.

CONCLUSION

In conclusion, this method has proven to be a useful addition to the many DNA isolation protocols that are now available for plants. Its versatility, speed, and low cost have made it the procedure of choice in our lab and elsewhere.

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