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Methods in ENZYMOLOGY

Volume 395

Molecular Evolution: Producing the Biochemical Data, Part B

Edited by

Elizabeth Anne Zimmer, Ph.D
Eric Howard Roalson, Ph.D

Methods in Enzymology

Volume 395

MOLECULAR EVOLUTION: PRODUCING THE BIOCHEMICAL DATA

Part B

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Volume 395

Molecular Evolution: Producing the Biochemical Data

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Elizabeth A. Zimmer

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NATIONAL MUSEUM OF NATURAL HISTORY
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Section I

Comparing Macromolecules: Exploring Biological Diversity

Subsection A
Accessing the Templates

[1] Isolation of DNA from Plants with Large Amounts of Secondary Metabolites

By ELIZABETH A. FRIAR

Abstract

Many plant species have high contents of polysaccharides, polyphenols, or other secondary metabolites that can interfere with DNA extraction and purification. These contaminating compounds can lead to poor DNA yield and prevent access by modifying enzymes, such as restriction endonucleases and *Taq* polymerase. A number of factors, including choice of plant tissue, tissue preparation, and modifications of the extraction buffer, can help in DNA extraction for difficult plant species. This chapter presents some of the DNA extraction protocols developed for various plants.

Introduction

The tissues of many plant species contain large amounts of secondary compounds. These compounds may be derived from various biosynthetic pathways and play many roles in the biology of the organism. Secondary metabolites may play important roles in plant defense, as chemotoxins to potential fungal, insect, or vertebrate predators. Other plant secondary compounds, particularly complex polysaccharides, play an important role in plant osmoregulation and protection from desiccation. Consequently, complex polysaccharides can make up a high percentage of tissue wet weight in cacti and other plants adapted to very dry environments.

The presence of these plant secondary compounds can make DNA extraction problematic. Some of them will coprecipitate with DNA during extraction and inhibit further enzymatic modification of the DNA, including restriction endonuclease digestion and polymerase chain reaction (PCR) (Guillemaut and Marechal-Drouard, 1992). Large amounts of complex polysaccharides can make extraction of usable DNA impossible, rendering the aqueous portion of the extraction too viscous to allow for

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efficient separation of DNA from the contaminating polysaccharides. These polysaccharides can also tightly adhere to the DNA, preventing access by modifying enzymes (de la Cruz *et al*., 1997).

A number of methods have been developed to dilute, selectively precipitate, or inactivate the contaminating substances. For removal of polysaccharides, higher concentrations of cetyltrimethylammonium bromide (CTAB), either in the initial extraction step or in a stepwise fashion over several steps, help to selectively precipitate DNA. Higher concentrations of CTAB and the addition of polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP) can help to remove polyphenols. Additionally, some protocols suggest the use of ascorbic acid, diethyldithiocarbamic acid (DIECA), and 2-mercaptoethanol to reduce oxidation and prevent DNA degradation.

The relative success of different protocols appears to be largely taxon dependent; what may work for one taxon may not work for another. Here, I present a number of protocols optimized for several taxonomic groups. It may be necessary to try several approaches to achieve high yields of clean DNA from a given species.

Tissue Choice

The choice of tissue for DNA extraction can be critical. Typically, the younger the tissue, the lower the amounts of secondary compounds. Thus, very young leaf tissue or newly germinated seedlings may be good choices for DNA extraction. Additionally, flower buds, petals, epidermal layer, or other soft tissues may be superior, particularly for plants such as cacti in which stem tissue is highly mucilaginous. However, at least one protocol has been developed to extract DNA from bark or young wood of woody species, allowing for identification of cultivars or rootstocks in winter condition (Cheng *et al*., 1997; see below for protocol).

Tissue Preparation

Typically, the more finely ground the tissue before DNA extraction, the better the extraction of DNA from the contaminating compounds. This can be accomplished by fine grinding in a mortar and pestle of tissues frozen in liquid nitrogen. Highly mucilaginous tissues, however, may become too hard to grind by hand. In those cases, it is possible to coarse chop frozen tissues in a blender or food processor, followed by hand grinding. Lyophilization of plant tissues can also aid in DNA extraction and minimize the amount of contaminating substances. In this case, the plant tissue can be

lyophilized and stored, then ground to a fine powder in a bead mill, and extracted using a standard CTAB extraction protocol.

DNA Yield

Yield of DNA from several of these extraction methods can be quite low. Barnwell *et al.* (1998) found that using the same protocol, pea shoots yielded 300 μg of DNA per gram of fresh tissue, but *Sedum telephium* yielded only 20 μg/g of fresh tissue. Some of this difference can be attributed to the high water content of many succulent plants. In addition, some DNA will be inevitably lost because it has been complexed with polysaccharides or polyphenols and lost during the extraction procedure. The more purification steps requiring differential precipitation or extraction, the more DNA will be inevitably lost. Thus, protocols that can successfully remove contaminants from genomic DNA in as few steps as possible will provide the best yields.

It should also be noted that samples yielding low amounts of DNA should be redissolved in distilled water rather than TE buffer, if PCR applications will be used downstream. If yields of DNA are low, the normal practice of dissolving the pellet in TE for a stable stock solution and diluting an aliquot in water for PCR may lead to levels of ethylenediaminetetraacetic acid (EDTA) in the PCR stock sufficient to inhibit *Taq* polymerase activity.

RNA Removal

All of the protocols presented here will coprecipitate DNA and RNA. Retention of contaminating RNA in DNA extractions can inflate measures of DNA concentration, because of the overlapping fluorescence peaks. RNA may also complicate downstream PCR protocols by selectively amplifying shorter complementary DNA (cDNA) copies rather than the desired genomic copies of genes. RNA contamination may be particularly problematic in random amplified polymorphic DNA (RAPD) protocols.

RNA can be removed by incubation with RNase A, either after the nucleic acids have been extracted or by inclusion of RNase A in the initial extraction buffer. RNA may also be removed by differential precipitation using lithium chloride. This protocol will remove larger RNA fragments, and smaller RNA species (e.g., tRNAs) may remain in the solution with the genomic DNA. (See detailed protocols in the following sections.)

Methods: Isolation of DNA

Method 1: Isolation of DNA from Mucilaginous Tissues (Large Preparation)

1. Preheat 2× CTAB buffer (2% CTAB, 1.4 *M* NaCl, 0.2% 2-mercaptoethanol, 20 m*M* EDTA, 0.1 *M* Tris–HCl, pH 8.0) to 60°. Aliquot 25 ml of preheated buffer into a 50-ml conical tube for each DNA extraction.
2. Grind about 5 g of fresh tissue to a fine powder using one of the methods described earlier in the section ''Tissue Preparation.''
3. Immediately brush ground powder into the preheated isolation buffer and place in a 60° water bath.
4. Incubate at 60° for 30 min.
5. Add an equal volume of chloroform:isoamyl alcohol (24:1) to each tube and agitate for 10 min. Adequate mixing of both layers is vital at this stage.
6. Centrifuge tubes at 6000 rpm for 5 min.
7. Transfer supernatant to a new 50-ml tube, being careful not to disturb the white layer between phases.
8. Add 2/3 volume of ice-cold isopropanol to supernatant and gently rock tube to precipitate DNA.
9. Collect DNA by pelleting or spooling. Pellet DNA by centrifugation for 2 min at 4000*g*. Spool by collecting long strands of DNA using a glass rod or pipette with a bent tip (a DNA hook can be made by gently heating the narrow end of a disposable glass pipette and bending it into a hook shape). Spooling selectively extracts high-molecular-weight DNA and reduces the amount of coprecipitating contaminants.
10. Transfer pellet to one or two 1.5-ml Eppendorf tubes filled with 0.75 ml of 60° 2× CTAB buffer. Use of two tubes allows for better agitation and increases yield. Mix well.
11. Incubate tubes at 60° for 30 min, mixing occasionally.
12. Add an equal volume of chloroform:isoamyl alcohol (24:1) and agitate well for 10 min.
13. Centrifuge in a Microfuge at maximum speed for 5 min.
14. Transfer supernatant to a new 1.5-ml tube.
15. Add 2/3 volume of ice-cold isopropanol and gently rock tubes to precipitate DNA.
16. Collect DNA by centrifugation in a microcentrifuge at maximum speed for 3 min.
17. Carefully pour off isopropanol, rinse with 70% ethanol, and centrifuge at maximum speed for 2 min.

18. Pour off ethanol and dry pellet thoroughly in a vacuum centrifuge or vacuum oven.

19. Resuspend pellet in 200 μl of TE or diH_2O.

Method 2: Isolation of DNA Using Starch Digestion

1. Mix 2× CTAB (2% CTAB, 1.4 *M* NaCl, 20 m*M* EDTA, 0.1 *M* Tris–HCl, pH 8.0) buffer with 1% w/v caylase (Cayla, Inc.). Just before using, add 1% 2-mercaptoethanol.

2. Cut approximately 1 g of leaf tissue into small pieces and then grind in liquid nitrogen. Add 7 ml of the CTAB mixture to the powder and mix to form a slurry. Pour the slurry into a 15-ml polypropylene tube.

3. Cap the tube loosely and incubate at 65° for 30–60 min without shaking.

4. Add 5 ml of chloroform:isoamyl alcohol (24:1) to each tube, cap tightly, and mix well.

5. Centrifuge tubes at 7000*g* for 10 min.

6. Using a wide-bore pipette, transfer the supernatant to a clean, labeled 30-ml Corex tube. Add 5 ml of ice-cold isopropanol. Cap the tube and swirl gently to precipitate the DNA. Place at −20° for at least 1 h.

7. Centrifuge the tubes at 7000*g* at 4° for 30 min to pellet the DNA. Pour off the supernatant and dry pellets in a vacuum oven for 10 min.

8. Dissolve the pellet in 1.5 ml of distilled water. Place in a 37° water bath for 10–30 min, mixing the tube occasionally to dissolve the pellet. The pellet will probably not dissolve completely. Transfer pellet and solution to a 15-ml polypropylene tube.

9. Add 7 ml of CTAB to each tube (without caylase or 2-mercaptoethanol). Cap tubes loosely and incubate at 65° for 30 min.

10. Repeat steps 4, 5, and 6.

11. Centrifuge tubes at 7000*g* at 4° for 30 min. Add 5 ml of ice-cold EtOH-NH_4OAc (10 m*M* NH_4OAc in 76% EtOH) to each tube to pellet DNA. Swirl gently, then let stand at room temperature for 10 min. Pour off supernatant and dry the pellet in a vacuum oven for 10 min. Be careful not to pour off the pellet, because it may be loose.

12. Resuspend pellet in 0.2 ml of diH_2O or TE at 37° and transfer solution to a 1.5-ml Microfuge tube.

Method 3: DNA Isolation Using Nucleon PhytoPure Resin (Micropreparation)

1. Grind 0.1 g of frozen plant tissue in liquid nitrogen to form a fine powder.

2. Transfer powder to a 1.5-ml Microfuge tube.

3. Add 500 μl of CTAB extraction buffer 1 to each tube (500 μl 2× CTAB [0.1 *M* Tris–HCl, pH 8.0, 1.4 *M* NaCl, 20 m*M* EDTA, pH 8.0, 2% w/v CTAB, 1% w/v PVP-40, 1% w/v sodium bisulfite, 0.2% 2-mercaptoethanol], 2 μl of 20 mg/ml proteinase K, 2 μl 2-mercaptoethanol). Mix well by vortexing or by inversion.

4. Incubate tubes at 50° for 20–30 min, mixing every 5 min.

5. Transfer tubes to 65° and incubate another 15 min.

6. Remove tubes from water bath and incubate on ice for 2–3 min.

7. Add 500 μl of ice-cold 100% chloroform to each tube, then add 100 μl nucleon resin, using a wide-bore pipette tip. Invert tubes to mix, degas once, and then close lids tightly.

8. Gently shake or rock tubes for 15 min at room temperature.

9. Centrifuge tubes in a microcentrifuge at maximum speed for 10 min.

10. Transfer supernatant to a 2-ml tube. (Do not use a 1.5-ml tube, as the additional volume is necessary for optimal mixing.)

11. Add 1 ml of ice-cold 95% ethanol to each tube to precipitate DNA. Incubate tubes at −20° for at least 1 h or overnight.

12. Centrifuge tubes in a microcentrifuge at maximum speed for 10 min to pellet DNA.

13. Pour off supernatant.

14. Dry pellets in a vacuum centrifuge for 10–20 min.

15. Resuspend pellets in 150 μl of diH_2O and incubate at 37° for 10–20 min, mixing every 5 min.

16. Add 500 μl of CTAB extraction buffer 2 to each tube (500 μl 2× CTAB, 1% w/v caylase) and invert to mix. Incubate tubes at 65° for 30 min.

17. Remove tubes from water bath and place on ice for 3–5 min.

18. Add 500 μl of ice-cold 100% chloroform to each tube. Invert tubes to mix, degas once, and then close lids tightly.

19. Centrifuge tubes in a microcentrifuge at maximum speed for 10 min.

20. Transfer supernatant to new 2-ml tube.

21. Add 1 ml of ice-cold 95% ethanol to each tube, invert gently to precipitate DNA. Incubate tubes at −20° for at least 1 h. An overnight incubation is preferable.

22. Centrifuge tubes in a microcentrifuge at maximum speed for 10 min. Discard supernatant.

23. Wash pellet by adding 500 μl of 75% ethanol to each tube and rocking gently for 10 min.

24. Centrifuge tubes in a microcentrifuge at maximum speed for 5 min. Discard supernatant.

25. Dry pellet in a vacuum centrifuge for 10–20 min.