

EXTRACTION OF TOTAL CELLULAR DNA

[Although this method was developed for fresh, herbarium and mummified plant and fungal tissues, it has also been used to obtain DNA from mammals, fishes, slime molds, lichens, marine algae and bacteria.]

OBJECTIVE:

To purify high molecular weight DNA (>50-100 kb) so that it can be used in standard molecular biology procedures and experiments. During the process, proteins (including nucleases that destroy DNA and RNA) as well as polysaccharides (that inhibit some of the enzymes used in molecular biology) are removed from the DNA.

INTRODUCTION:

In general, extraction procedures for plant and fungal DNAs must accomplish the following:

1. The cell walls must be broken (or digested away) in order to release the cellular constituents. This is usually done by grinding the tissue in dry ice or liquid nitrogen with a mortar and pestle or a food grinder or in a microfuge tube. The tissue can be fresh, frozen, lyophilized or dried (as with herbarium specimens).
2. The cell membranes must be disrupted so that the DNA is released into the extraction buffer. This is accomplished by using a detergent, usually SDS (sodium dodecyl sulfate = lauryl sulfate) or CTAB (cetyltrimethylammonium bromide = hexadecyltrimethylammonium bromide).
3. The DNA must be protected from the endogenous nucleases. The detergents are also useful for this purpose, since they will denature and inactivate many such enzymes. Another common additive is EDTA (ethylenediaminetetraacetic acid), which is a chelating agent that binds magnesium ions, generally considered necessary cofactors for most nucleases (although one nuclease in wheat is known to be stimulated by EDTA). In addition, the buffer/tissue mixture is emulsified with either chloroform or phenol to denature and separate the proteins from the DNA.
4. Shearing of the DNA should be minimized. DNA in solution can be broken by exposure to turbulence (e.g., by being drawn quickly through a small orifice). Typically, DNA 30-100 kb in length can be obtained without great care being taken.
5. The time between thawing of the frozen, pulverized tissue and its exposure to the extraction buffer should be minimized to avoid nucleolytic degradation of the DNA.

There is one other major consideration associated with the isolation of DNA from plants and fungi that is not encountered with most other organisms. Enzyme-inhibiting polysaccharides are often present in "purified" DNA. Most extraction methods have employed the expensive and time-consuming cesium chloride density gradient technique to eliminate the polysaccharides (e.g., Bendich et al. 1980, Murray & Thompson 1980, Taylor & Powell 1982). Some methods have been reported that do not utilize density gradients, but have been described for only a few species and tissue

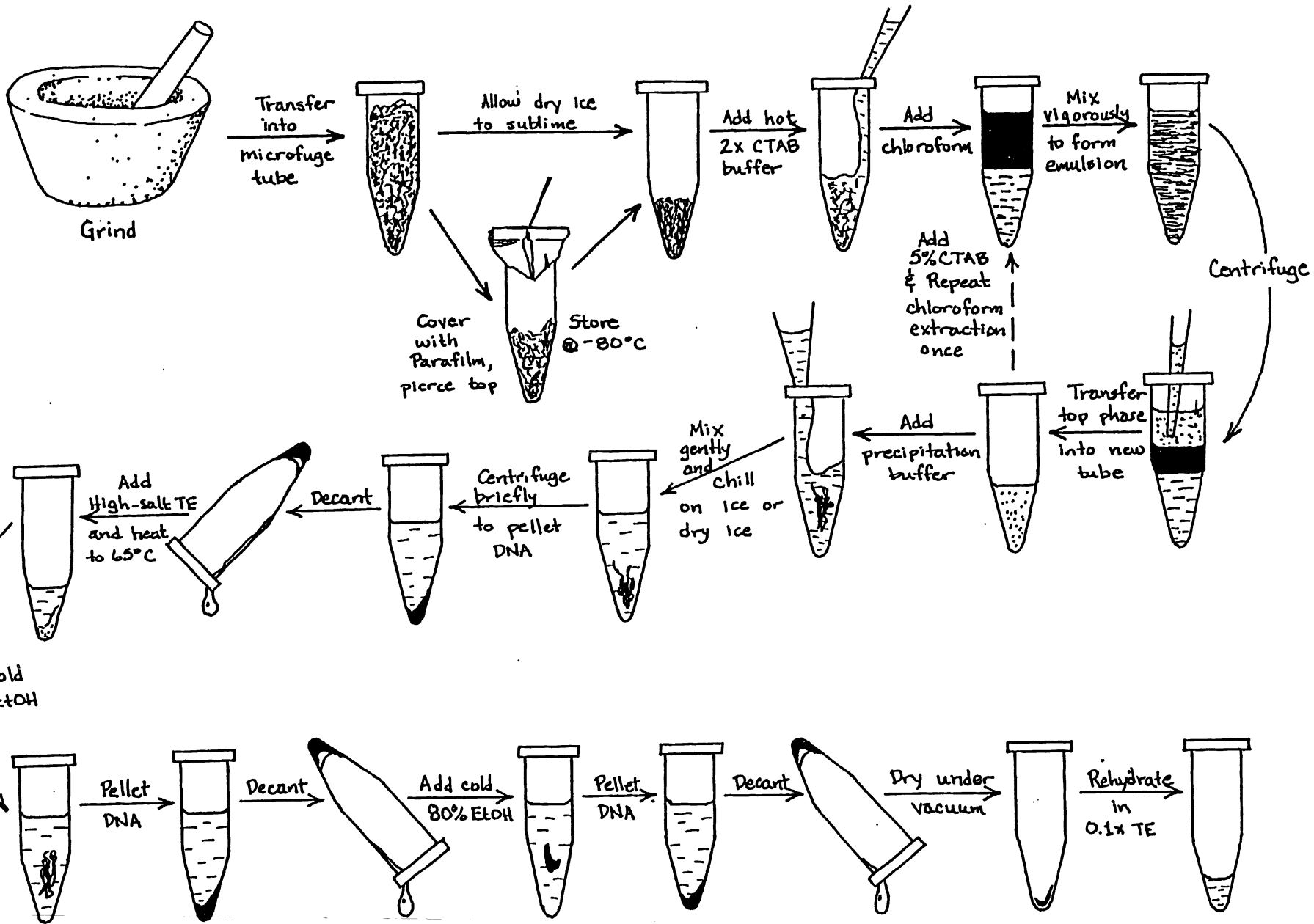
types (Jones & Boffey 1984, Saghai-Marroof et al. 1984, Zimmer & Newton 1982). The method presented here is based on the CTAB nucleic acid extraction procedures of Murray and Thompson (1980), Taylor and Powell (1982), Rogers and Bendich (1985, 1988, 1994), Rogers, et al. (1989), and Shivji et al. (1992) that make it possible to extract high molecular weight DNA without the use of expensive equipment and/or time-consuming procedures. Because fewer manipulations of the DNA are involved there is generally less chance for degradation to take place.

The basis for the separation of polysaccharides from nucleic acids is their differential solubilities in the presence of CTAB. In most cases only three disposable microcentrifuge tubes are required for all operations from tissue homogenization to DNA of sufficient purity to be digested by most restriction endonucleases. Tissues as small as individual ovules and embryos, small pieces of an individual plant, cultured hyphae or mycorrhizae can be used. In addition, DNA can be obtained from milligram amounts of herbarium and mummified tissues. Extraction of DNA from isolated cell organelles can also be accomplished using this method (which will do in a later exercise). It has been used to extract DNA from over 100 types of tissue from more than 200 species. The original method (Rogers & Bendich 1985, 1988, 1994) is presented here with a few refinements and is described for tissue amounts smaller than 500 mg, although it can be easily scaled up by using larger containers.

STEPS IN THE PROCEDURE:

1. Grind dry ice to a powder in a mortar and pestle.
2. Grind tissue with dry ice with the chilled mortar and pestle (or in a chilled microfuge tube, using a stainless-steel rod, ground to fit the bottom of the tube - this can also be attached to an electric drill for more efficient cell breakage).
3. Transfer the tissue/dry ice mixture into a pre-chilled (with dry ice) microfuge tube (if it is not already in one). Allow the dry ice to sublime away at room temperature for several minutes or at -80 °C for several hours (or overnight). [NOTE: The ground tissue can be stored at -80 °C for extended periods of time, up to several years.]
4. When the dry ice has sublimed away, add hot (65 °C) CTAB buffer. If you started with fresh tissue, add about an equal volume (to the approximate volume of the tissue) of 2X CTAB buffer. Place the tube into a 65 °C water bath for a minute or so. [With some fresh, dried, or lyophilized tissues more liquid is needed. If this is the case, add 1X CTAB buffer (dilute the 2X buffer) until the correct consistency is attained.] The mixture should be gooey and viscous, like thick mud. If it is too thick, it will be cakey. Add more buffer in stages until the correct consistency is attained. If the mixture is too watery at this stage, start over with a new sample since later precipitation steps will be inefficient and the yield of DNA will be very low. The correct consistency is extremely important at this stage. If the mixture is too thick, it will be difficult to remove the supernatant after centrifugation. If it is too dilute, precipitation will be adversely affected. Once you are familiar with the correct consistency you should be able to extract DNA from almost any tissue using this method.

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5. After placing the tube in the 65 °C water bath for at least 3-5 min (or up to 20 min) add one volume (equal to the current total volume of the contents of the tube) of chloroform/isoamyl alcohol (a 24:1 solution, the chloroform extracts proteins, the isoamyl alcohol decreases foaming of the emulsion). Mix thoroughly to form a complete emulsion. This is extremely important. When a complete emulsion is formed the two separate phases will not be seen (i.e., no chloroform phase should form at the bottom of the tube until centrifugation).
6. Centrifuge in a microfuge for 30 seconds (@ 11,000 g).
7. Transfer the supernatant solution from the top (aqueous) phase into a new microfuge tube. Measure the volume with the micropipeter when you are making the transfer. Discard the lower (chloroform) phase.
8. Add 1/5 volume of the 5% CTAB solution and mix.
9. Perform another chloroform/isoamyl alcohol extraction as in steps 5-7 (do not repeat step 8).
10. If a low yield is expected, add 10-50 µg of RNA (e.g., yeast tRNA) as a carrier to aid in the later precipitation steps.
11. Add an equal volume of CTAB precipitation buffer and mix gently and watch for a precipitate to form.
12. If a large stringy precipitate or a very cloudy solution forms, centrifuge for 10-30 seconds in the microfuge. If a faintly cloudy solution forms or the solution remains clear, the yield is low, centrifuge for 1-5 minutes. Discard the supernatant.
13. Rehydrate the pellet in high-salt TE buffer. This is usually done in stages. Add 20 µl for small pellets, 50 µl for moderate pellets, and 100 µl for large pellets. Heat in a 65 °C water bath for at least 5 min, occasionally flicking the tube to rehydrate the pellet. If the pellet does not completely dissolve, add more high-salt buffer. Do not add more than a total volume of 500 µl. If it still refuses to dissolve, remove the supernatant and proceed with it to the next steps.
14. Add two volumes of cold 95% or 100% ethanol to the solution and mix gently.
15. Centrifuge for 5 min (or longer if no precipitate is observed). Discard supernatant.
16. Add one volume (equal to the volume of the solution in step 14) of cold 80% ethanol, mix and centrifuge for 3-5 min. Discard the supernatant.
17. Dry in a desiccator to 20-30 min or until all of the liquid has evaporated.
18. Rehydrate the pellet in 0.1X TE. Add 20 µl to small pellets, 50 µl for moderate pellets and 100 µl or more to very large pellets.
19. Test with restriction enzymes and treat with RNase if desired.

SOLUTIONS:

2X CTAB buffer:

2% CTAB (w/v)
100 mM Tris (pH 8.0)
20 mM EDTA (pH 8.0)
1.4 M NaCl
1% PVP (polyvinylpyrrolidone) M_r 40,000 [optional]

5% CTAB solution:

5% CTAB (w/v)
0.7 M NaCl

CTAB precipitation buffer:

1% CTAB (w/v)
50 mM Tris (pH 8.0)
10 mM EDTA (pH 8.0)

High-salt TE buffer:

10 mM Tris (pH 8.0)
1 mM EDTA (pH 8.0)
1 M NaCl

1X TE buffer:

10 mM Tris (pH 8.0)
1 mM EDTA (pH 8.0)

Transfer RNA:

10 mg/ml in 0.1X TE

RNase stock solution:

1 mg/ml RNase A
100 U/ml RNase T₁

[The solution should be heated to 90-100 °C for at least 10 min, then allowed to cool at room temperature for about 30 min to destroy any DNases. It can then be kept frozen in aliquots until needed. Repeated thawing and freezing does not appear to affect the activity of these RNases.]

95% EtOH:

80% EtOH:

(Dilute 95% EtOH)

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