

ORGANELLAR DNA ISOLATION

INTRODUCTION:

The study of organellar DNA requires that DNA samples highly enriched for the organellar component be obtained. In the case of animals, fungi, etc., mitochondria are the only other cell inclusion that contain DNA, other than the nucleus. Plant cells, on the other hand, contain mitochondria as well as plastids (chloroplasts, amyloplasts, etc.) that contain their own DNAs. While all three components (chloroplasts, mitochondria and nuclei) contain different DNA sequences, some transfer of DNA between organelles has occurred during evolution, since parts of the chloroplast and mitochondrial genomes are found in the nucleus. Additionally, parts of the chloroplast genome have been found in the mitochondria. Most cells generally contain more than one of each organelle. Each separate organelle often contains hundreds of copies of its genome. Because of the multiplicity of the organelles and the multiplicity of their genomes, the amount of organellar DNA per cell often exceeds 20% of the total DNA per cell. In cases where the percentage is high the restriction enzyme bands can be seen in total DNA preparations.

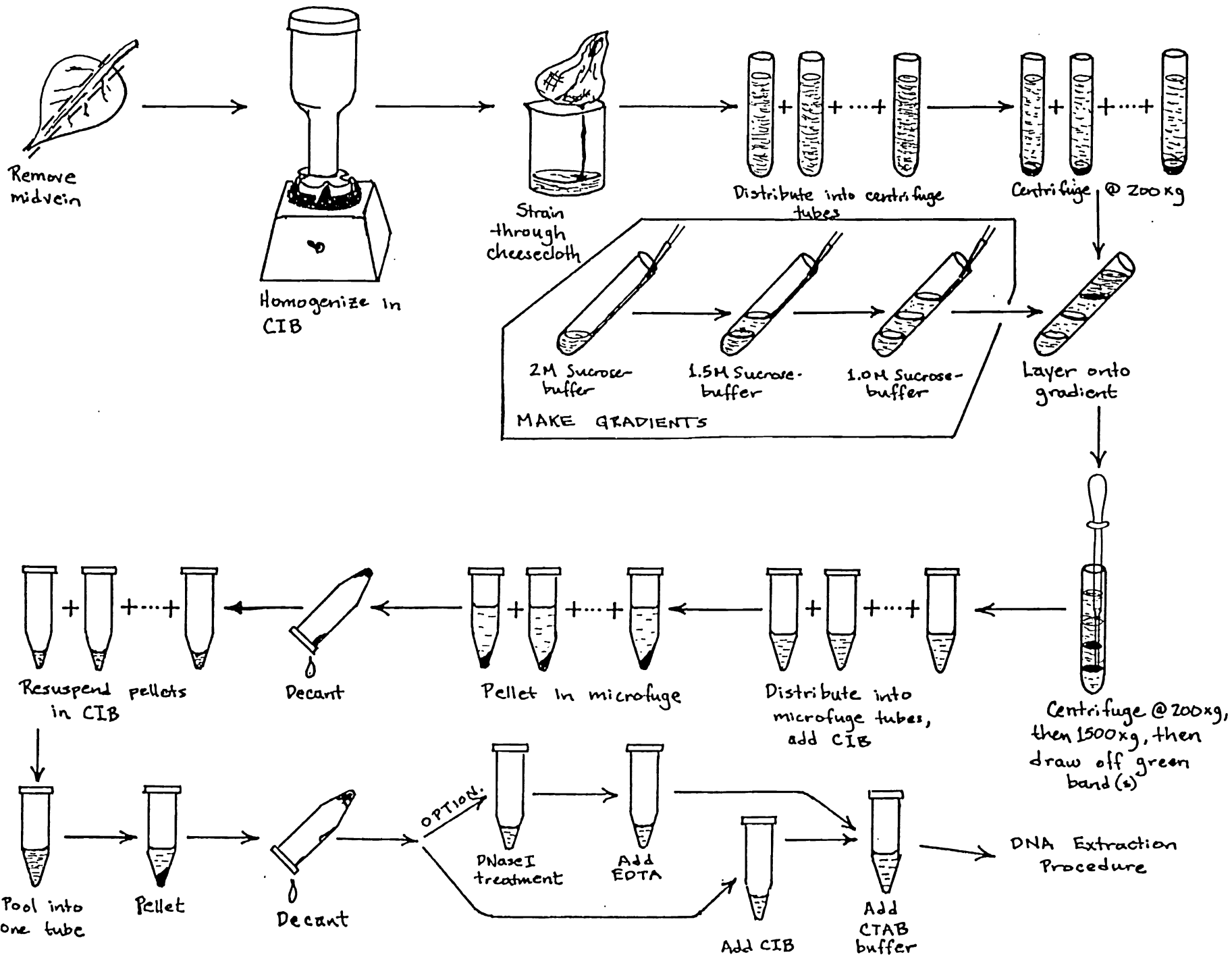
Organelle genome sizes are relatively small compared to the nucleus (around 150 kb for plastids, between 15 and 2500 kb for mitochondria versus 7000 to 9×10^7 kb for nuclei). Because of this, banding is clearly seen when restriction digests of the organellar DNA is observed on agarose gels. This is in contrast to the pattern exhibited by nuclear DNAs of higher eukaryotes that are usually so large and complex that only a few bands (corresponding to high and middle repetitive sequences) can be seen over a background smear of DNA (low copy number sequences). Although the organellar DNA can sometimes be observed on restriction digests of total DNA preparations, in order to accurately study these genomes, enrichment of the organellar DNA must be accomplished. All methods of enrichment employ low and high speed centrifugation and the use of density gradients of one sort or another. Initially, a low speed centrifugation step is used to pellet the nuclei and starch grains. Next, the supernatant is loaded onto a gradient, or the organelles are pelleted and resuspended before being loaded onto a gradient. Two main types of media are used for gradients: sucrose and Percoll. Sucrose is the easier and cheaper to use than Percoll. The resolution and purification can often be just as good with sucrose. After the organelles have been isolated (using sucrose or Percoll) a good deal of contaminating nuclear (and other) DNA is present in the solution and adhering to the outside of the organelles. Further purification can be achieved in two ways. The first is to treat the intact organelles with DNase I. The enzyme cannot pass through the organelle membranes and will thus digest any DNA that is outside of the intact organelles. Any broken organelles, however, will allow access to the enzyme and will have their DNA digested. So this method is also a test for the condition of the organelles. Before lysing the organelles for extraction of the DNA, the DNase I must be inactivated, generally by using a high dose of EDTA. Generally, the DNA that is then extracted is highly enriched organellar DNA. Of course in the case of plants, unless the initial gradients were accurately carried out, both mitochondria and chloroplasts may be present. Occasionally, after the DNase I treatment, CsCl gradients are used to further purify the DNA. Bisbenzamide (also known as Hoechst 33258) is used in the gradients to accentuate the base composition differences. This dye associates with AT-rich portions of the DNA and causes regions of DNA with higher proportions of AT to be buoyed up in the gradient. For higher plants, plastid DNA has nearly the same base composition as most of the nuclear DNA, so that the method cannot be utilized for many plants. For algae, the chloroplast DNA can be separated using this method. Mitochondrial DNA for most higher plants contains a higher proportion of GC than does

nuclear DNA, and does not take up as much of the dye as the bulk of the nuclear DNA regions. Because of this, mitochondrial DNA can often be separated from nuclear DNA using this method.

We will be using sucrose density gradients to enrich for spinach chloroplasts (or, if desired, mushroom mitochondria). You will also have the option of treating the organelles with DNase I to eliminate much of the nuclear DNA contamination. After the organelles are prepared, a CTAB DNA extraction will be performed.

STEPS IN THE PROCEDURE:

1. Each group grind 20 g of spinach leaves (that have had their midveins removed) in 50 ml of cold chloroplast isolation buffer (CIB) in a blender with a small container ("semi-micro"). Grind in three 10 second bursts. Between each burst, check to see that all of the tissue is being homogenized in the buffer.
2. Over a clean 100 ml beaker, strain the homogenate through four layers of cheesecloth.
3. Pour the homogenate into two or more centrifuge tubes (Falcon 2059, without caps). Centrifuge using a swinging bucket rotor (rotor number 215) at $200 \times g$ in a centrifuge for 5 minutes. [On an IEC CENTRA-4B table model centrifuge this is about 1100 RPM, or a setting of about 35-37 on the dial. On the larger model IEC centrifuges, the same g -force is achieved with a dial setting of 20-25. All of these values are approximate, and in most cases achieving the exact g -force is not absolutely necessary.]
4. Keep this on ice while each person prepares a gradient as follows:
 - a. Using either a Pasteur pipette or a P1000 Pipetman, pour 3 ml of the 2.0 M sucrose-buffer solution into the bottom of a 2059 Falcon tube.
 - b. Tilt the tube at an angle and gently layer 3 ml of the 1.5 M sucrose-buffer on top of the 2.0 M sucrose-buffer layer. You should be able to clearly see the interface between the layers.
 - c. Again with the tube at an angle, gently add a layer of 3 ml of the 1.0 M sucrose-buffer solution.
 - d. Finally, gently layer on 4 ml of the spinach leaf homogenate.
5. Centrifuge the gradients at 1100 RPM ($200 \times g$) for 5 minutes, then at 3250 RPM ($1500 \times g$, about 60 on the dial) for 30 minutes.
6. Remove the gradient from the centrifuge and transfer the green band into several 1.5 ml microfuge tubes, leaving room in the tubes to add about 0.5 ml of the CIB (without sucrose).
7. Add 0.5 ml CIB to each tube and centrifuge in a microfuge for 1-3 minutes.
8. Pour of the supernatant and add 100 μ l of CIB to each tube.
9. Resuspend each pellet and pool all of the suspensions into one tube.
- 10A. Centrifuge for 1 minute, pour off the supernatant and add back 50 μ l of CIB.



10B. [OPTIONAL] To get rid of most of the contaminating DNA:

- a. Instead of resuspending in CIB, resuspend the pellet in 50 μ l of the DNase I buffer (which also contains sorbitol to keep the osmotic pressure at a spot that will keep the plastids intact).
- b. Add 0.5 μ l DNase I (1 U/ μ l) and incubate at room temperature for 15 min.
- c. Add 15 μ l 0.5 M EDTA and mix completely.

11. Freeze the pellet at -20 °C.

12. To isolate the DNA add 50 μ l of hot (65 °C) 2X CTAB extraction buffer. Heat to 65 °C and proceed through the CTAB DNA Extraction Procedure from step 4. Remember to add the carrier tRNA in step 10.

SOLUTIONS:

Chloroplast isolation buffer (CIB)

- 0.33 M sorbitol
- 5 mM MgCl₂
- 1 mM DTT
- 5 mM sodium phosphate, monobasic
- 5 mM sodium phosphate, dibasic
(adjust pH to 6.8)

1.0 M sucrose-buffer

- 1.0 M sucrose in CIB

1.5 M sucrose-buffer

- 1.5 M sucrose in CIB

2.0 M sucrose-buffer

- 2.0 M sucrose in CIB

DNase I buffer

- 0.4 M sorbitol
- 10 mM Tris (pH 7.5)
- 50 mM MgCl₂

DNase I enzyme solution

- 1 U/ μ l (depending on the source 1 U = 400 mg)