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Green DNA

Simple isolation, restriction and electrophoresis of chloroplast DNA

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Introduction

Soon after the rediscovery of Mendel's Laws, patterns of inheritance were observed that suggested the existence of extra-chromosomal genes in plants. It was not until 1962, however, that definitive proof that chloroplasts contain DNA was provided. In most species, chloroplast DNA (ctDNA) is inherited through the maternal line and has a low mutation rate. It can therefore provide important clues about evolution.

Between 10–100 chloroplasts are found in each photosynthesizing cell, and each chloroplast contains 50–100 copies of the ctDNA. Some 10–20% of a plant's total DNA is found in the chloroplasts.

Like that of plasmids and mitochondria, ctDNA is circular; typically it is 120–150 kb in length, encoding ~80 proteins. Many of the proteins that chloroplasts require to function are, however, encoded in the nuclear DNA. These include, for example, the enzymes needed for replication of the ctDNA. To date, the ctDNA of about 25 species has been fully sequenced.

Aim

School students are able to prepare crude extracts of DNA from a variety of plant sources, using simple procedures [1]. Since the advent of pre-aliquotted restriction enzymes, inexpensive electrophoresis systems and safe staining methods, it has also become common for school students to carry out restriction digests and gel electrophoresis of lambda DNA [2, 3].

Unfortunately, when students isolate nuclear DNA from plants and try to obtain visible bands using gel electrophoresis after a restriction digest, the results are often disappointing. This is because of the great length and numerous restriction sites found in plants' nuclear DNA. The many fragments formed produce a 'smear' on the gel rather than distinctive bands.

This paper describes a simple but effective method for the isolation of relatively pure ctDNA. In contrast to plant nuclear DNA, ctDNA is small and has few restriction sites. Consequently it is possible to digest this DNA to produce clear bands following electrophoresis. This technique presents ample opportunities for open-ended investigations.

The isolation method described here is a simplified version of that developed by Bookjans *et al* [4]. This method relies upon a high-salt extraction buffer, which minimises nuclear contamination — unlike similar extraction media that contain sucrose as an osmoticum for keeping chloroplasts intact.

Equipment and materials

Needed by each person or group

For extracting chloroplast DNA

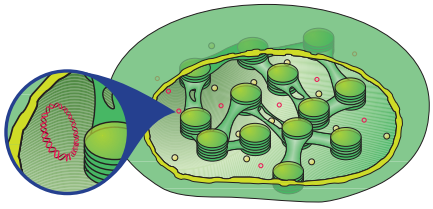
- 20–30 g of fresh leaves (about 2 large handsful). Chinese radish, pea and turnip leaves are suitable, although the results from peas can be variable. Kale, spinach, salad leaves *etc*. from supermarkets may not give good results; you need fresh young leaves without too much fibrous material. Frozen material gives poor results because freezing disrupts chloroplast structure, so very few intact chloroplasts are obtained.
- Tris-EDTA-NaCl extraction buffer, 60–90 ml for each extraction
- Detergent-NaCl solution, 10 ml for each extraction
- 100 µl of protease (about 2 drops) for each extraction, *e.g.*, *Novozymes Neutrase*®
- Ethanol, 3 ml for each extraction (at least 80% ethanol is required; this should be ice-cold — see *Safety*, below)
- Sterile water, 100 µl for each extraction
- Filter funnel
- Beaker, 100 ml
- Gauze or muslin, about 200 mm x 800 mm, folded into four
- Centrifuge tubes, 15 ml, 7 for each extraction
- Microcentrifuge tubes (1.5 ml), 4 for each extraction
- Plastic Pasteur pipettes ('Pastettes'), 2 for each extraction
- Plastic stirring rod *e.g.*, Swizzle stick
- A supply of crushed ice in an insulated container *e.g.*, in a polystyrene cup
- Paper towel
- Access to a kitchen blender, a stopclock, a centrifuge, a microcentrifuge, a water bath or incubator maintained at 60 °C and a water bath or incubator maintained at 37 °C

For the restriction digest

- Micropipette(s) or the equivalent to dispense 20 and 100 µl volumes
- Dried restriction enzymes *Bam*HI or *Hin*dIII containing 10 units of enzyme, or the wet equivalent together with an appropriate buffer
- Holder for tubes during incubation (this can be made by cutting holes with a cork borer in a piece of closed-cell foam *e.g.*, foam sleeping mat)
- Access to a water bath or incubator, maintained at 37 °C

For the gel electrophoresis

- Gel electrophoresis equipment and power supply
- Micropipette(s) or the equivalent to dispense 2 and 25 µl volumes
- Agarose solution (0.8% in TBE buffer)
- Bromophenol blue loading dye
- TBE running buffer, pH 8.5
- Azure A solution (0.08% in 20% ethanol) for staining the gel
- **OPTIONAL:** 1 kb DNA 'ruler' or ladder



DNA (not to scale here) is found in the stroma of chloroplasts.

Procedure

Because DNA sticks to glass, it is important to use plastic pipettes, tubes *etc*. for much of this procedure. It is also important to keep all solutions and equipment on ice, unless otherwise stated. This helps to prevent degradation of the DNA.

A Isolation of chloroplast DNA

1 Place 20–30 g of fresh leaves in a kitchen blender. Add 3 times the volume of the starting mass of Tris/EDTA/NaCl extraction buffer (*e.g.*, add 90 ml of buffer to 30 g of leaves). Blend for 10–15 seconds.

2 Filter the leaf material through four layers of guaze or muslin into a beaker and keep the filtrate. *The guaze filters out the cellular and leaf debris — only the chloroplasts pass through.*

3 Centrifuge the filtrate at 3 000 rpm for 5 minutes, then discard the supernatant. If the pellets are not densely-packed you must take great care not to tip them away! *The pellets contain chloroplasts. The supernatant will be light green in colour because some of the chloroplasts will have been broken and lost their chlorophyll. The thylakoid membranes liberated on breakage will be be broken up further on blending, and some will remain in the supernatant.*

Fig. 1

Fig. 2

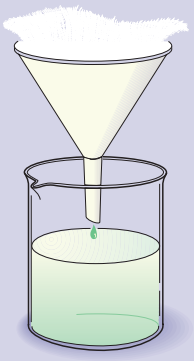


Fig. 3

4 **OPTIONAL:** Using a pipette, gently resuspend the pellets, each in 5 ml Tris-EDTA-NaCl extraction buffer, then combine the suspensions and centrifuge for 5 minutes at 3 000 rpm. Remove and discard the supernatant as before. *This second centrifugation helps to remove any remaining cell debris (other than chloroplasts) from the suspension.*

5 Combine all the chloroplast pellets and resuspend them in 10 ml Detergent-NaCl solution, then incubate at 60 °C for 15 minutes. *This resuspension can be difficult because the solution is so viscous. Although you can be fairly rough at this stage (the aim is to rupture the chloroplasts), try to avoid excess frothing of the detergent. If you started with more than 30 g of leaves, add more Detergent-NaCl solution in proportion.*

6 Cool the suspension quickly on ice, add 100 µl (about 2 drops) of protease and incubate at 37 °C for 10 minutes. *This denatures nucleic acid-degrading enzymes and any other proteins that might interfere with the later steps.*

Fig. 4

Fig. 5

Fig. 6

7 Cool the suspension on ice to below 10 °C then centrifuge for 5 minutes at 3 000 rpm. *You should obtain a yellowish/green supernatant above a dark green pellet of chloroplast debris.*

8 Using a pipette, remove the supernatant carefully into a new tube, then cool it on ice. *The supernatant contains the ctDNA.*

9 Use a pipette to transfer ice-cold ethanol onto the supernatant. Ensure that the liquids do not mix. Use slightly more ethanol than you have chloroplast extract. Leave the tube on ice for at least 10 minutes. *White threads of DNA will appear at the junction of the two layers, then be carried into the ethanol layer.*

10 With care, pipette the upper (ethanol) layer with the ctDNA into 2–4 microcentrifuge tubes. Spin the ctDNA to the bottom of the tubes in a microcentrifuge at 10 000 rpm for 5 minutes. *A light brown pellet should be visible in each tube.*

11 Pour away the ethanol and leave the tubes upturned on a paper towel overnight to drain. *It is important that all the alcohol is allowed to evaporate. If the DNA is not to be used immediately, it may be frozen and stored for several months.*

Fig. 7

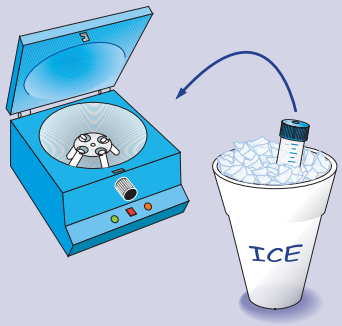


Fig. 8

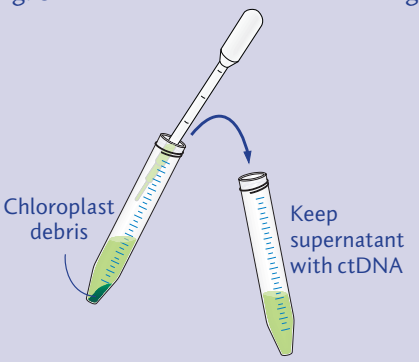


Fig. 9

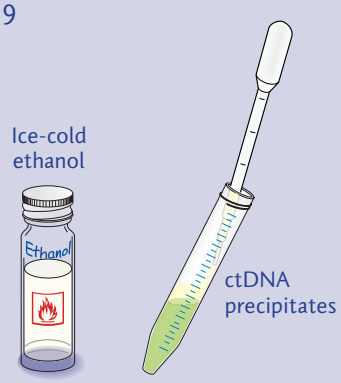
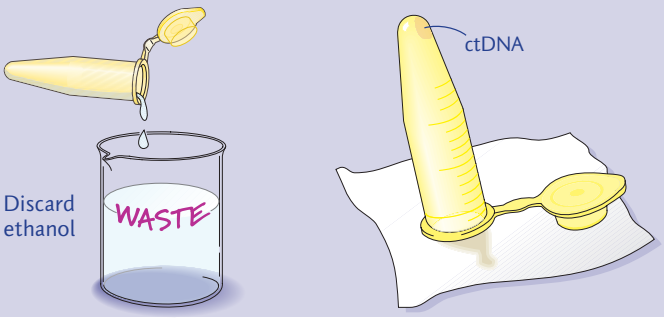


Fig. 10

Fig. 11



B Restriction digest

12 Reconstitute and combine the ctDNA from all the tubes in a small amount of distilled water. *For a starting quantity of 30 g of leaves, a total volume of ≤100 µl of water is sufficient.*

13 Add 20 µl of this solution to a tube containing 10 units of *Bam*HI or *Hin*dIII (buffered appropriately if you are using wet enzymes). *Note: With the species tested (Chinese radish [Raphanus sativus], turnip [Brassica campestris ssp. rapa] and pea [Pisum sativum]), 'digestion' of ctDNA with EcoRI gave too many bands to be useful.*

14 Incubate the tube(s) at 37 °C for 30–45 minutes (but no longer) in a water bath or incubator. *Incubation for too long may result in 'star' activity, where the restriction enzymes cut the DNA at places other than their 'recognition' sites. The 'digested' samples can be stored, frozen, until needed for electrophoresis.*

Fig. 12

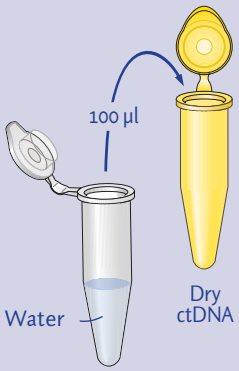


Fig. 13

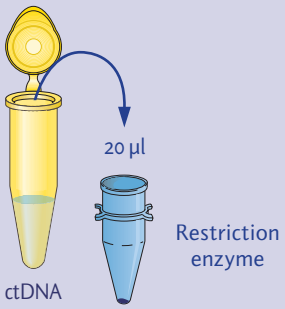
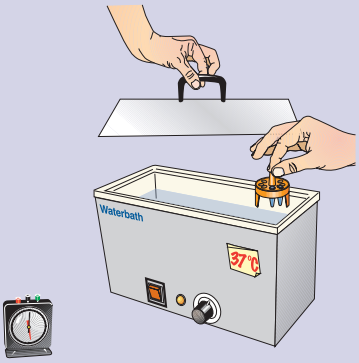


Fig. 14



C Gel electrophoresis

15 Add 2 µl of Bromophenol blue loading dye to each tube of digested ctDNA.

16 Load ~20 µl samples into wells in an electrophoresis gel. **OPTIONAL:** Add a 1 kb DNA 'ruler' or ladder to one or more wells. This will enable the size of the ctDNA fragments to be estimated.

17 Electrophorese until the loading dye reaches about 10 mm from the end of the gel. *If you are using the NCBE minigels, this will take approximately 3½ hours at 27 V. With conventional equipment, electrophoresis should take 30 minutes at 80 V.*

Fig. 15

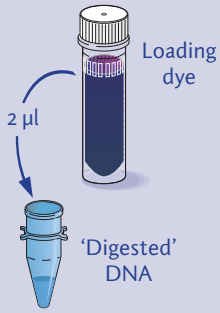


Fig. 16

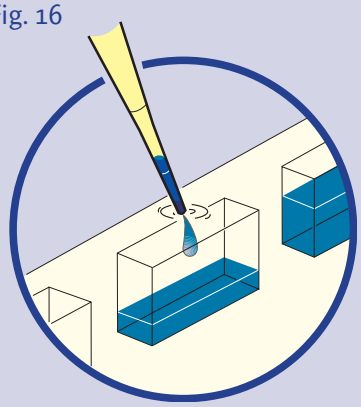
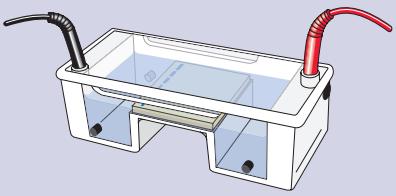


Fig. 17



18 Disconnect the power supply and stain the gel with Azure A solution for *exactly* 4 minutes.

19 Pour off the stain and allow the coloured bands of ctDNA to develop. *Depending upon the thickness of the gel, this will take between 20 minutes and 6 hours. After several hours, the loading dye will diffuse through the gel, so that the smaller fragments of ctDNA can be seen even more clearly.*

Fig. 18

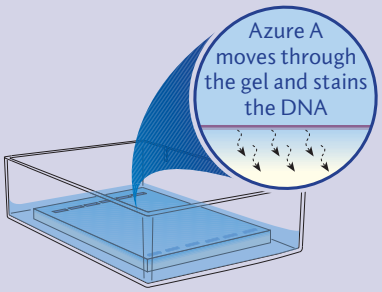
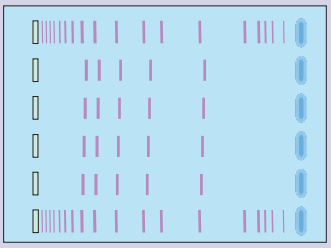
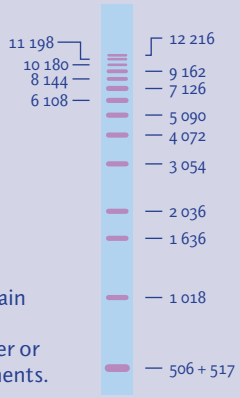


Fig. 19





Above: A stained gel. The patterns you obtain will not match those shown here.
Right: Fragment sizes in a typical 1 kb ladder or 'ruler' allow comparison with ctDNA fragments.

Specimen results

*Figures 20–22 show digests of chloroplast DNA from Chinese radish (Raphanus sativus), turnip (Brassica campestris ssp. rapa) and pea (Pisum sativum) respectively. **B** indicates digests with BamHI; **H**, digests with HindIII and **U**, uncut DNA. The bands of greater intensity are due to restriction fragments of the same size — but from different parts of the ctDNA — running in the same places. This is usually due to restriction fragments derived from the inverted repeat region present in ctDNA from most higher plants (except peas). The best results were obtained with seedlings of about three weeks old.*

To obtain these photographs, ethidium bromide was incorporated into the gels at a concentration of 10 µg/ml, although this is not recommended for use in schools — see Safety. The gels were 'run' at 80 V for 30 minutes. A UV-transilluminator was used to visualise the DNA and the photographs were taken with a digital camera.

Fig. 20

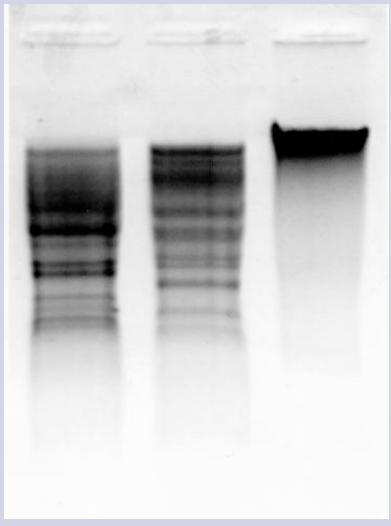


Fig. 21

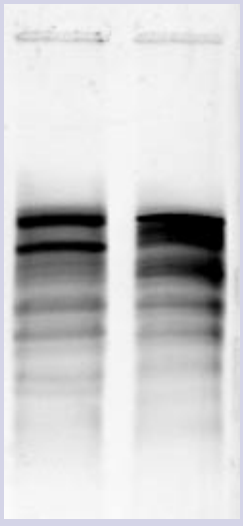
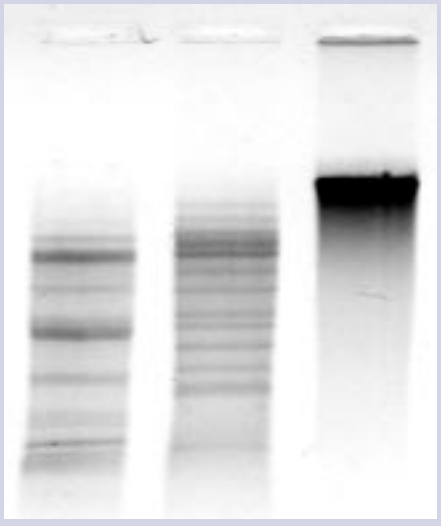


Fig. 22



Safety

To produce clear photographs for this publication, ethidium bromide was used to stain the gels shown in Figures 20–22. Ethidium bromide should not be used in schools unless an appropriate risk assessment has been undertaken and local regulations permit its use. Safer dyes, such as Azure A and Nile blue sulphate will produce excellent results, however, and are recommended for use in schools [5, 6].

Avoid inhaling the Tris, borate or EDTA powders used to make up the buffers. Wear a mask over your nose and mouth. All of these chemicals are irritants and can cause harm through physical contact and inhalation.

Molten agarose can scald, so care should be exercised when pouring the gels.

Most freezers are not spark-proof. Consequently, you must ensure that any ethanol placed in a freezer is in a sealed, vapour-tight container. An alternative to using a freezer is to stand the sealed bottle of ethanol in ice for several hours before use.

For more information about safety in schools when working with DNA, teachers in the UK should consult *Topics in Safety* [7].

Preparation

The leaves must be freshly-picked, with the petioles and stalks removed. Semi-wilted tissue gives poor results.

The Detergent-NaCl solution must be made up freshly.

All solutions and equipment should be cooled well beforehand. The ethanol must be chilled thoroughly before use.

If required, electrophoresis gels may be cast before a lesson to save time. They can be stored with a little TBE buffer over them and tightly wrapped, in a fridge, for up to a week.

Timing

The extraction procedure takes about 1½ hours, after which the DNA can be stored frozen until needed. Allow 45 minutes for incubation of the DNA with the restriction enzymes (if necessary, the gels can be prepared while this is happening). The electrophoresis itself takes about 3½ hours at 27 volts and 30 minutes at 80 volts. The gels can be stained in about 10 minutes, although results will not be seen clearly until 15 minutes to 3 hours later (depending upon the thickness of the gel).

Troubleshooting

Many washing-up liquids are not suitable for this work. They may extract the lipids so poorly from the cell membranes that DNA is not extracted. The author has found that *Fairy Liquid* (*Procter and Gamble*) gives good results.

Some people suggest that the yield of chloroplast DNA may be improved by leaving plants in the dark for 36–48 hours before starting the extraction procedure. The effectiveness of this 'destarching' procedure depends upon the species used however and is, in reality, unlikely to be effective.

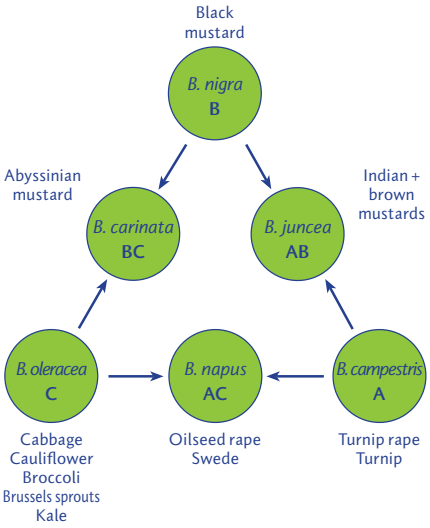


Fig. 23
Nuclear genomic relationships in Brassica. *Adapted from Erickson* et al. *(1983).*

Further investigations

In extracts of total leaf DNA [1] faint but discrete bands can sometimes be seen within the 'smear' of DNA. Do these correspond to bands of ctDNA? Try running total DNA and ctDNA in adjacent wells to investigate this.

Chloroplast DNA is usually inherited through the maternal line. A hybrid cross and its two parents could be investigated [8]. Brassicas are appropriate (Figure 23) although with the restiction enzymes *Bam*HI and *Hind*III the restriction patterns obtained may not be sufficiently distinctive to reveal the origin of the ctDNA.

Other fast-growing leafy plants — lettuce, spinach or cress could also be tested. Take care, however, if you use 'cress' or 'sprouting leaves' that are sometimes sold for salads, because they are often a mixture of species.

Suppliers

Many of the items required for this activity, including *Novozymes Neutrase*®, dried restriction enzymes and electrophoresis apparatus, are available from the NCBE, The University of Reading, Whiteknights, Reading RG6 6AP, The United Kingdom. http://www.ncbe.reading.ac.uk

An inexpensive microcentrifuge suitable for school use is available from Teaching Resources, Middlesex University, Unit 10, Lea Road, Waltham Cross EN9 1AS, The United Kingdom. http://www.mutr.co.uk

References

1 *Investigating plant DNA. Student's guide* by Dean Madden [Ed.] (1998) Reading: National Centre for Biotechnology Education. Second edition.

2 *DNA Science. A first course in recombinant DNA* by David Micklos and Greg Freyer (1990) New York: Cold Spring Harbor Laboratory Press. ISBN: 0 89278 411 3.

3 *The Lambda DNA protocol. Student's guide* by Dean Madden (2000) Reading: National Centre for Biotechnology Education. Third edition. ISBN: 0 7949 1371 2.

4 Bookjans, G., Stumann, B.M. and Henningsen, K.W. (1984) Preparation of chloroplast DNA from pea plastids isolated in a medium of high ionic strength. *Analytical Biochemistry* **141**, 244–247.

5 *Illuminating DNA* by Dean Madden (2000) Reading: National Centre for Biotechnology Education. ISBN: 0 7049 1370 4. *Page 39 of this publication gives details of methods of staining DNA on electrophoresis gels, using safer alternatives to ethidium bromide.*

6 Adkins, S. and Burmeister, M. (1996) Visualization of DNA in agarose as migrating colored bands: Applications for preparative gels and educational demonstrations. *Analytical Biochemistry* **240**, 17–23.

7 Delpech, R. and Madden, D. (2001) 'Working with DNA'. In *Topics in Safety* (Third Edition) pp. 99–105. Hatfield: Association for Science Education. ISBN: 0 8635 7316 9.

8 Erickson, L. R., Straus, N.A. and Beversdorf, W.D. (1983) Restriction patterns reveal origins of chloroplast genomes in *Brassica* amphiploids. *Theoretical and Applied Genetics* **65**, 201–206.

Web sites

Nicotiana tabacum plastid, complete genome (NCBI database)

http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?db=Genome&gi=13349

This site gives a genome map of tobacco chloroplast DNA (the first chloroplast genome from a higher plant to be sequenced).

Photosynthesis — an introduction

http://gened.emc.maricopa.edu/bio/bio181/BIOBK/BioBookPS.html

This site give basic information and diagrams suitable for students' studies.

Photosynthesis and the Web

http://photoscience.la.asu.edu/photosyn/photoweb/default.html

This site leads to individual pages on different aspects of photosynthesis.

Botany online — The Internet Hypertextbook

http://www.biologie.uni-hamburg.de/b-online

This comprehensive and authoritative site has good explanatory material on chloroplast DNA, although the translation from German makes it a little difficult to follow in places.

Acknowledgements

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Professor John Gray of the Department of Plant Sciences at Cambridge University kindly gave detailed assistance during the preparation of the manuscript.

Recipes

For extracting chloroplast DNA

Tris-EDTA-NaCl extraction buffer, pH 8.0

Used to extract the chloroplasts

Makes 1 litre
May be stored indefinitely at room temperature

- 6.06 g Tris (hydroxymethyl) aminomethane (Tris base)
- 9.31 g Ethylene diamine tetraacetic acid (EDTA, disodium salt)
- 73.05 g Sodium chloride

Add the above ingredients to 700 ml deionised or distilled water. Stir to dissolve. Make up to 1 litre. Adjust to pH 8.0 with dilute HCl.

Detergent-NaCl solution

Used to lyse the chloroplasts

Makes 100 ml
Make up freshly, immediately before it is required

- 10 g Sodium chloride
- 10 ml washing-up liquid (*e.g., Procter & Gamble Fairy Liquid*)

Add distilled or deionised water to the above materials, making up to 100 ml in total. Stir gently to dissolve.

For the gel electrophoresis

TBE (Tris-Borate-EDTA) buffer, pH 8.5

Used for making up agarose gel and as the electrophoresis buffer

Makes 1 litre of 10× concentrate
May be stored indefinitely at room temperature

- 1 g Sodium hydroxide
- 108 g Tris (hydroxymethyl) aminomethane (Tris base)
- 55 g Boric acid
- 7.4 g Ethylene diamine tetraacetic acid (EDTA, disodium salt)

Add the materials listed above to 700 ml of deionised or distilled water. Stir to dissolve. Make up to 1 litre with deionised or distilled water.

Note: Once made up, 1 volume of this TBE concentrate should be diluted with 9 volumes of distilled water before use.

Agarose gel

For separating small fragments of nucleic acids

Makes 100 ml of 0.8% agarose solution
May be stored indefinitely at room temperature

- 0.8 g DNA electrophoresis-grade agarose
- 100 ml TBE buffer (1×)

Add the agarose powder to the TBE buffer. Heat in a boiling water bath or microwave oven to melt the agarose. Less than a minute at full power in a 940 W oven is sufficient to melt 100 ml of gel. The container used to hold the molten agarose must not be sealed, but lightly covered with plastic film that has been punctured with one or two small holes. Swirl the gel half-way through the heating cycle to ensure that it is thoroughly mixed. Adjust the volume of agarose solution after microwaving by adding distilled water to make up the mass lost through evaporation. Once molten, the agarose solution can be kept in this state at 55–60 °C in a water bath.

Ensure that the agarose solution is mixed well before casting gels.

CAUTION!
Hot, molten agarose can scald and so it must be handled with care. It is advisable to let the molten agarose cool until it is comfortable to handle before pouring the gel.

For the gel electrophoresis

Bromophenol blue loading dye

Used for loading DNA fragments into the electrophoresis gel

Makes 100 ml
May be stored indefinitely at room temperature

- 0.25 g Bromophenol blue
- 50 g Sucrose
- 1 ml 1 Molar Tris (pH 8)

Add the ingredients listed above to 60 ml of deionised or distilled water. Stir to dissolve. Make up to 100 ml with deionised or distilled water.

Azure A solution

Stain for nucleic acids

Makes 50 ml of 2× concentrate
May be stored indefinitely at room temperature.

- 0.08 g Azure A
- 50 ml ethanol (40% aqueous solution)

Add the Azure A powder to 50 ml of 40% ethanol. Stir to dissolve.

CAUTION!
The concentrated DNA stain is flammable and must not be used near naked flames or other sources of ignition. The stain bottle must be kept closed to prevent evaporation of the solvent. When diluted to its working concentration (that is, 0.04% in 20% ethanol), the stain presents no serious safety hazard, although care should be taken to avoid splashes on the skin or eyes *e.g.*, wear protective gloves and glasses. Used stain may be diluted with water and washed down the drain.