National Centre for Biotechnology Education | Science and Plants for Schools

University of Reading

The PCR and Plant evolution

Student's guide

1.1

Chloroplast DNA

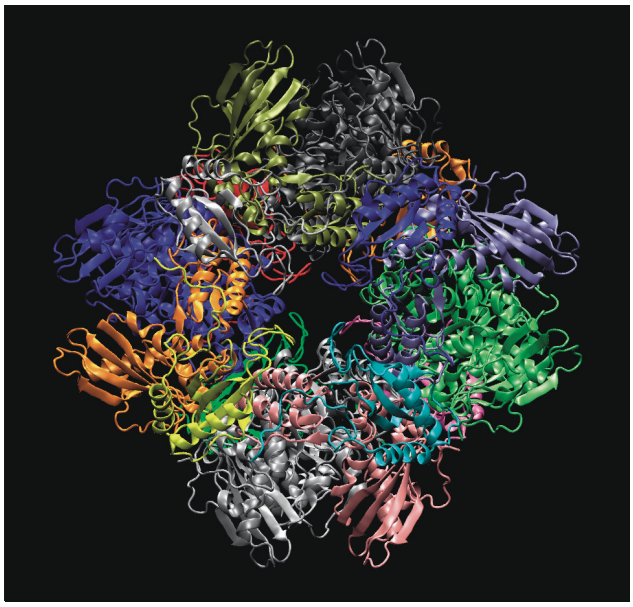
Gene regions provide a molecular guide to evolution

A revolution in the garden

In 1998, an international group of nearly a hundred plant scientists published a revolutionary proposal. After more than two centuries of classifying plants on the basis of their appearance, botanists began grouping flowering plants according to similarities in their genetic material, DNA.

The new system proved controversial and threw up some surprises. For instance, botanists used to think that the tropical papaya was a close relative of the passionflower. According to the DNA evidence, however, the papaya turns out to be a 'cousin' of the cabbage, and roses are more closely related to nettles than had previously been thought. A result of this research was the reordering of the plants within botanic gardens worldwide — a botanical 'Year Zero'.

The reclassification project was led by Mark Chase at the Royal Botanic Gardens, Kew (in London) and two colleagues: Kåre Bremer of the University of Uppsala in Sweden and Peter Stevens, then at Harvard University in the USA. The work had taken several years to complete. The *Angiosperm Phylogeny Group*, as the team became known, reclassified the world's flowering plants into 462 families. Five years later, using improved molecular data and computer analysis, the number of flowering plant families was reduced to 457.



The Angiosperm Phylogeny Group compared genes encoding a large subunit of the enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco). This model shows the eight large and eight small subunits from which Rubisco is made. The enzyme is needed for the carbon-trapping process of photosynthesis, and the gene for the large subunit, codenamed rbcL, is found in the DNA of chloroplasts. Variations in the DNA sequence of the gene enabled the APG scientists to propose a new 'family tree' for flowering plants.

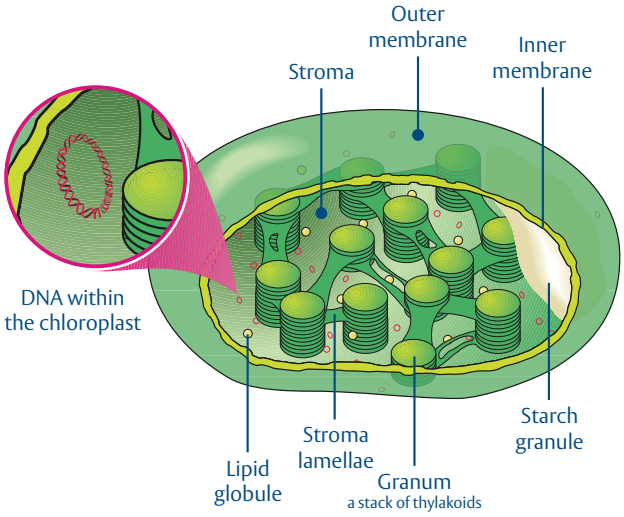
DNA evidence shows that the tropical papaya (Carica papaya) is a close relative of the humble cabbage.

Molecular methods

At the end of the eighteenth century the Swedish naturalist Carl von Linné, known as Linnaeus, attempted a systematic classification of plants. Since then, botanists have grouped plants into the different families according to similarities in their shapes and structures — the number of petals, the shape and organisation of a plant's leaves, vascular system, embryo, seeds and so on.

Traditional classification tended to reflect evolutionary relationships, but because natural selection produces similar-looking solutions to life's challenges, identifying the true underlying relationships was little more than guesswork. Now, by applying the techniques of molecular biology, a more accurate picture has begun to emerge. How has this been done?

An essential task is to identify DNA sequences that vary between species. Gene regions that are too variable will not, however, reveal the genetic similarities that are needed to create a family tree. Many gene regions have appropriate rates of change and are therefore suitable for studying plant evolution, but these regions are often rare and hard to work with. Fortunately chloroplasts contain their own plentiful supply of DNA that is ideal for evolutionary studies.



Internal structure of a chloroplast, showing the location of the DNA.

Chloroplasts have DNA

Soon after the rediscovery of Mendel's Laws in 1900, unexpected patterns of inheritance were noticed in plants. These observations hinted that plants might have extra genes that were not part of their chromosomes. It was not until 1962, however, that clear proof that chloroplasts contain their own DNA was provided.

Between 10–100 chloroplasts are found in each green plant cell, and each chloroplast contains 50–100 copies of the chloroplast DNA (cpDNA). In total, 10–20% of a plant's DNA is found in the chloroplasts. Like that of plasmids and mitochondria, the DNA of chloroplasts is circular: typically it forms a ring about 120–150 thousand base-pairs (120–150 kilobases, or kb) in length, encoding about 80 proteins. The cpDNA of many plant species has now been sequenced.

In most species, cpDNA is inherited through the egg cells and does not undergo recombination, which limits the amount of variation found. cpDNA does, however, show nucleotide insertion, deletion and substitutions.

High-resolution sequencing techniques are required to detect small variations *within* a plant species, but it is possible, even with simple equipment, to detect larger differences in the cpDNA *between* major plant groups.

The right genes

Several different genes within cpDNA have been used by botanists to trace genetic relationships between plants. The APG team originally used one of the Rubisco genes, *rbcL*. cpDNA also contains genes that encode a complete set of transfer RNAs (tRNAs). tRNA genes are highly conserved — they have not changed much during the course of evolution. Identical nucleotide sequences can therefore be found in the cpDNA of most higher plants. While there are common regions in the genes of chloroplasts, the stretches of DNA *between* them, especially in a non-coding stretch of cpDNA, can vary greatly. Such regions have a higher frequency of mutation and demonstrate relatively high rates of evolutionary change, revealing genetic differences between populations of plants that have been genetically isolated for some time. It is these genetic 'signatures' that are examined in this practical investigation.

Comparison of cpDNAs

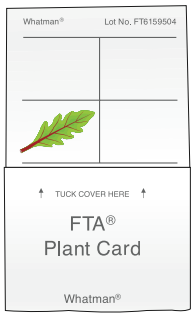
This investigation uses the polymerase chain reaction (PCR) to make copies of the highly-variable DNA that lies between the conserved tRNA genes in chloroplasts.

The PCR produces millions of copies of DNA which can be seen as bands on an electrophoresis gel. Electrophoresis also allows the lengths of the variable cpDNA from different species to be compared. The distance moved on the gel by the DNA will vary according to the lengths of the DNA fragments: shorter fragments will travel further than longer DNA fragments on the same gel.

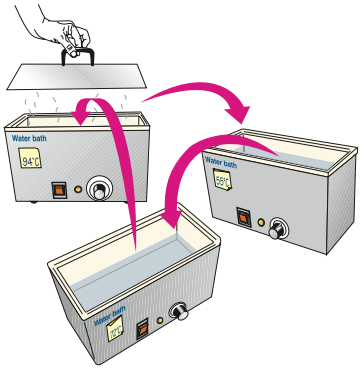
If the DNA from two different plants moves the same distance on the gel, the sizes of DNA fragments made by the PCR are the same for those plants. This may indicate that they are genetically similar and are therefore closely related. Species that yield differently-sized fragments may be more distantly related. From these results it is possible to infer evolutionary relationships.

The results obtained here are relatively crude, and they are derived from a single variable region of DNA alone. Many such regions must be used along with other techniques such as DNA sequencing, to build a more accurate picture of evolutionary relationships.

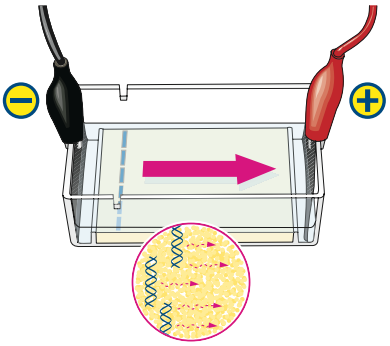
Summary of the protocol



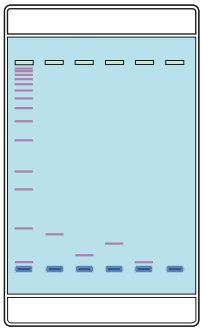
Collect DNA from fresh green plant tissue



Amplify the variable regions of the chloroplast DNA using PCR



Separate the amplified DNA fragments by gel electrophoresis



Related plants give DNA fragments of similar sizes

Making a haystack from a needle

Duplicating DNA — the polymerase chain reaction

A genetic 'photocopier'

The polymerase chain reaction (PCR) has been likened to a genetic 'photocopier'. From a small amount of biological material millions of copies of a chosen section of its DNA can be made quickly, accurately and automatically.

Although it was first devised for carrying out medical diagnostic tests, today the PCR has a wide range of uses ranging from forensic genetic fingerprinting and archæological investigations to tracing the mating and feeding habits of animal species.

A method called 'sexual PCR' or 'DNA shuffling' enables protein engineers to develop better enzymes for industrial use by mimicking the processes of evolution.

Without a clever variant of the PCR called 'cycle sequencing' the Human Genome Project would have been almost impossible — as would much of the molecular biology that is carried out in laboratories around the world. It is hardly surprising therefore that the PCR won for its inventor, Kary Mullis, a Nobel Prize for Chemistry in 1993. What is more surprising is the PCR's unusual origin.

A drive through the woods

The road to the PCR began on a moonlit evening in April 1983. In Kary Mullis's account of his invention, he describes how its two key features occurred to him while driving to his weekend retreat in California. Twice Mullis had to pull over and stop the car: once to calculate the number of copies of DNA that the process might generate; and a second time to sketch a diagram, showing how it might duplicate a specific stretch of DNA.

The idea was so simple that at first people refused to believe that it would work. No one could believe that it hadn't been tried before and found to be impractical for some reason. After Mullis's initial work, his colleagues at the Cetus Corporation, one of the first modern biotechnology companies, carried out extensive research to refine the method and eventually the process was patented.

Mullis was paid a bonus of US$ 10,000 for his invention. The PCR has become so important however, that Cetus was later able to sell the rights associated with the process to Hoffman-La Roche for US$ 300 million.

The breakthrough that made the PCR into a convenient laboratory technique was the isolation of DNA polymerase from Thermus aquaticus, a bacterium found in hot water springs. This enzyme (known as Taq polymerase) is stable at high temperatures and can therefore withstand the rigours of the reaction. In the computer-generated structure shown here, β-pleated sheets are coloured yellow, while the α-helices are magenta. A small fragment of the DNA that is being made is shown as a space-filling model.

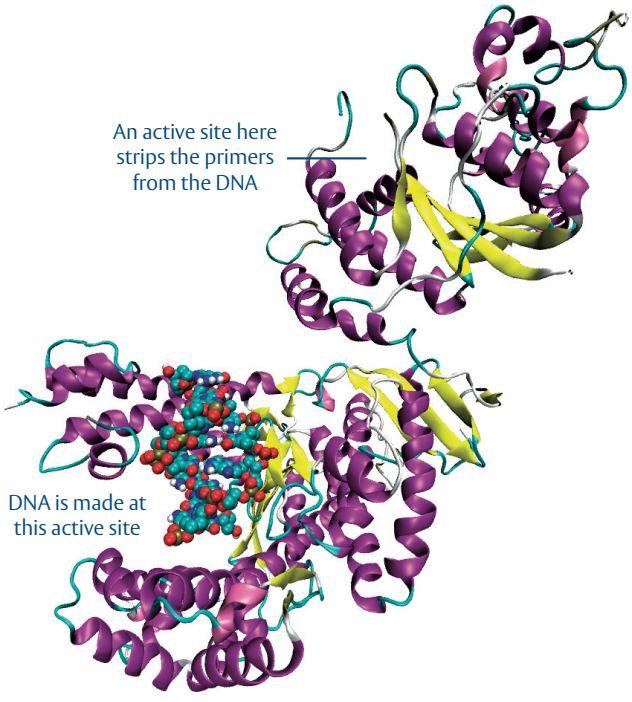
How the PCR works

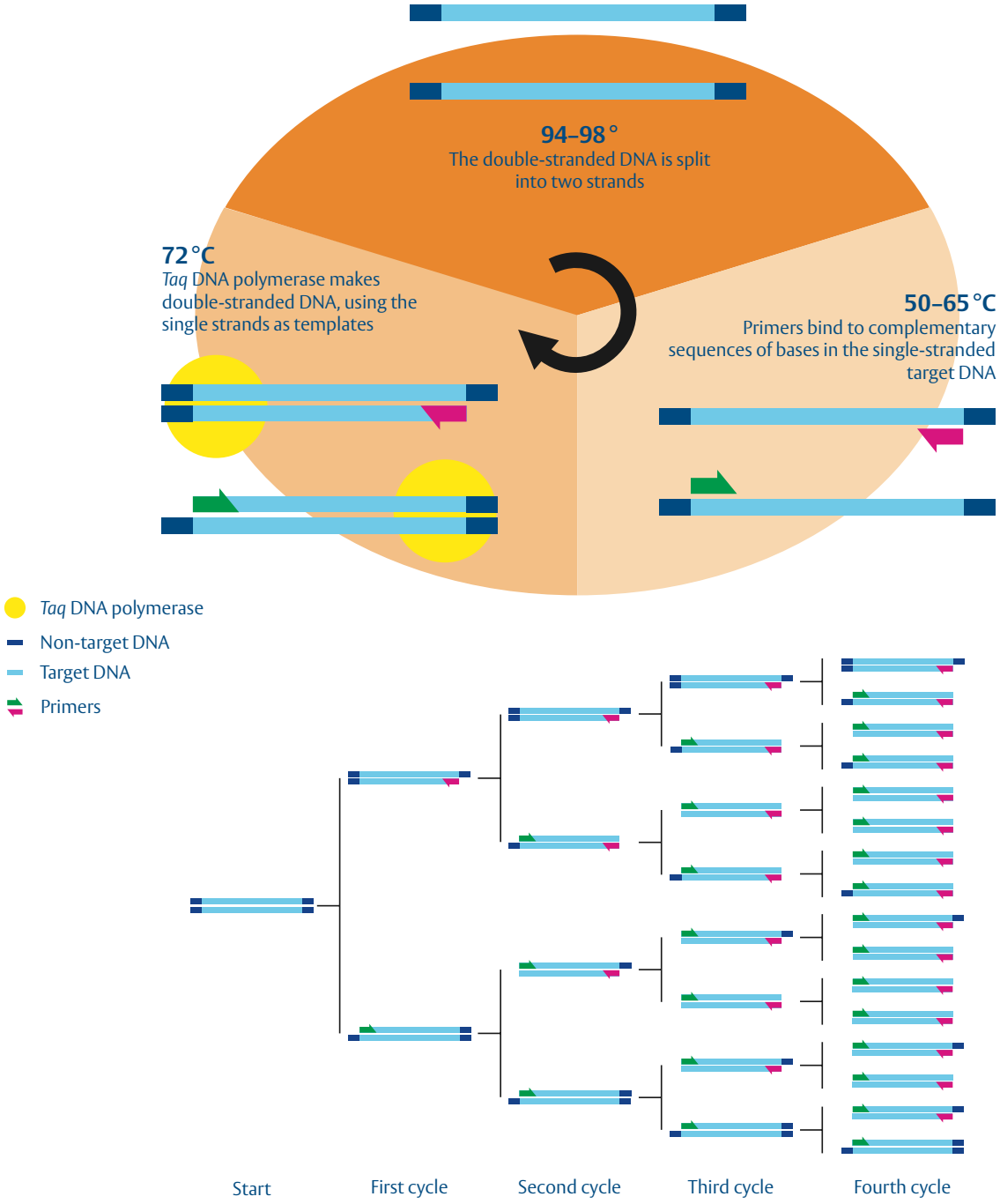
The modern method for PCR (a slightly modified version of Mullis's original idea) operates as follows:

The components

- Double-stranded DNA (the 'template') is extracted from the biological sample under study.
- Two single-stranded 'primers' are made that flank the specific stretch of DNA to be copied. These are generally 15–30 bases long, and are complementary to (in other words, they pair up with) the 5' end of each target DNA strand.
- The template DNA is mixed with the primers.
- Equal proportions of deoxyribonucleoside triphosphates (dNTPs) are added. dNTPs are the units from which DNA is made.
- Heat-stable *Taq* DNA polymerase (obtained from the hot-water bacterium *Thermus aquaticus*) is added to the reaction mix, together with Mg^{2+} ions that are required as a co-factor by the enzyme. This enzyme strings together dNTPs to make new DNA.

All of the components needed for the PCR (apart from the primers and the template DNA) can be dried into a small pellet in the correct proportions. Then, all that needs to be done is to add the primers and template DNA to a tube containing the pellet. This removes the need to dispense very small volumes of numerous reagents and improves the reliability of the process.





The process

Once the chemicals necessary for making DNA have been mixed, the PCR has three steps which are repeated 20–40 times:

Denaturation

The double-stranded DNA is split into two single-stranded templates by heating it to 94–98 °C.

Annealing

The mixture is cooled to 50–65 °C. The primers bind to the complementary strands of DNA by base-pairing.

Extension

Heating to 72 °C (the optimum temperature for *Taq* polymerase) encourages the synthesis of new DNA strands alongside the templates. This step doubles the number of DNA molecules present.

The process of heating and cooling is now repeated. With each cycle (lasting about 2 minutes) the number of copies of the DNA is doubled. After 30 cycles, 1,073,741,764 copies of the target DNA will have been made.

The first attempts at PCR were made using water baths held at three different temperatures. Soon the process was automated. The first PCR machine, made in 1985 with washing machine parts, was known affectionately as 'Mr Cycle'. This machine was only semi-automatic however, because before the discovery of temperature-stable *Taq* DNA polymerase, fresh enzyme had to be added with each cycle.

The PCR is a very sensitive method and in theory it can amplify a single DNA molecule. Scrupulous precautions are therefore often necessary to ensure that stray DNA molecules stay out of the reaction mixture.

Measuring small volumes

Three different devices allow you to dispense microlitre volumes of liquid accurately

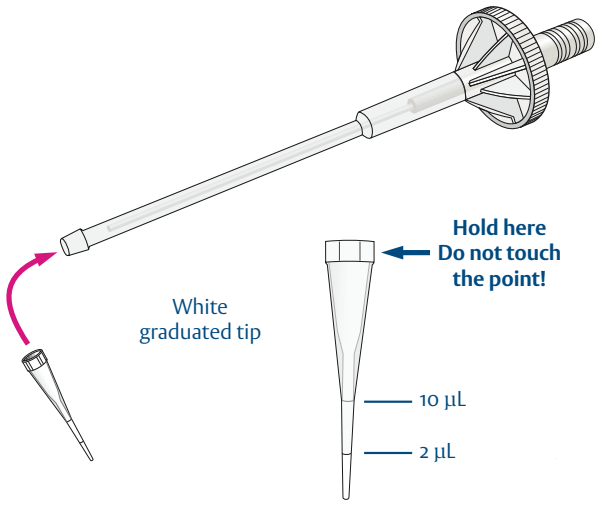
Syringes with graduated tips

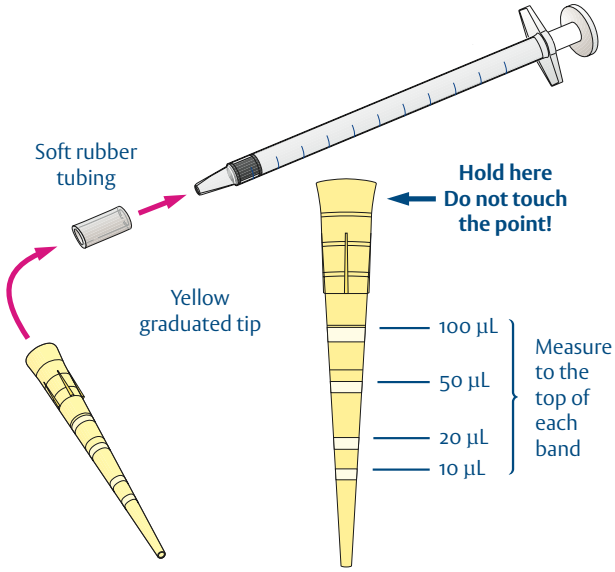
Two types of syringes — conventional 1 mL syringes and narrower 'microsyringes' — can be fitted with disposable graduated tips and used to measure small volumes. The white microsyringe tips are marked at 2 and 10 microlitres (µL). The larger yellow tips can be fitted to a 1 mL syringe and used to dispense volumes between 10 and 100 µL. To make an effective seal between the tip and the syringe body, it is necessary to use a short length of soft silicone rubber tubing that extends 1–2 mm beyond the nozzle of the syringe (if the tubing is cut too short, you will not be able to fix a tip onto the syringe).

When you use syringes like this, it is very important to draw liquid into the tip only, and to use the graduations on the tip as a guide. Ignore any markings on the syringe itself and take care not to draw liquid into the syringe barrel.

With both types of syringe, the best results will be obtained if you observe the following precautions:

- Never pull the plunger right out of the syringe — when the plunger is re-inserted the seal may be damaged;
- Before you load the tip, pull the plunger out a little (1–2 mm). This will give you some extra air with which to expel the last drop of liquid from the tip;
- When you dispense liquids, hold the syringe as near to vertical as possible, and at eye level so that you can see what you are doing;
- Remove the liquid from the point of the tip by holding it against the inner wall of the tube into which you are transferring the liquid;
- Do not touch the point of the tip with your fingers. There are proteases and DNases in your sweat which may contaminate and degrade the reactants.

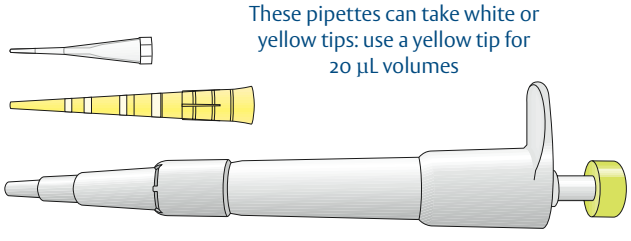




Fixed volume micropipettes

Fixed volume micropipettes can be used to measure small volumes with less than 1% error, but to achieve this accuracy you need to use them correctly. Firstly, the tip must be pushed firmly onto the pipette, so that it forms an airtight seal. Secondly, note that the pipettes have a double action. Use them as follows:

- Press the plunger down to the first stop;
- Place the tip into the liquid then *slowly* release the plunger, drawing liquid into the tip;
- To expel every last drop of liquid from the tip, press the plunger down hard to the second stop;
- After dispensing the liquid, lift the tip clear of the liquid before letting go of the plunger (otherwise the pipette will draw the liquid up again).



Volume
A microlitre is one millionth of a litre
1000 microlitres (µL) = 1 millilitre (mL)
1000 millilitres = 1 litre (L)

Choosing plant species

Research and select your test species carefully to obtain the most interesting results

Using the techniques described in this booklet, we have successfully amplified chloroplast DNA from a wide variety of plant species. These include many food plants that can be found on the shelves of supermarkets or greengrocers. Although the PCR primers are known to work for algae, liverworts, ferns, non-flowering and flowering plants, the FTA® card extraction method described here is not generally suitable for plants such as pine or cabbage that have tough or fibrous leaves that are difficult to crush.

Plants that are distantly-related will tend to give DNA fragments that differ most in size. It would be interesting to compare, for example, orchids (which are recently-evolved) with *Ginkgo* (which is similar to plants found in the fossil record 225 million years ago). Guidance for selecting plant species is provided by the web-based *Tree of Life* project: **http://tolweb.org/** and by the work of the *Angiosperm Phylogeny Group* (see page 2): **http://www.mobot.org/MOBOT/research/APweb/**

In our (limited) tests, DNA from none of the following plants could be extracted with the FTA® card method:

- 'Cress' (*Brassica napus* or *Lepidium sativum* seedlings);
- Cabbage and broccoli (both plants are the same species, *Brassica oleracea*);
- Thyme (*Thymus vulgaris*) or basil (*Ocimum basilicum*);
- Cucumber (*Cucumis sativus*) or courgette (*Cucurbita pepo*) skin — but we did not try the leaves.

The practical protocol

1 Collecting DNA from plants

a. Fold back the cover of the FTA® Plant Card. Take a fresh leaf or piece of plant material and place it on the card. *Ensure that the plant material does not extend outside one of the four boxes printed on the card.*

b. Close the cover over the plant material and, using a pestle, squash the leaf onto the card until moisture has soaked through to the *back* of the card. *Try not to allow the sample to spread outside the box printed on the card as this can contaminate adjacent samples.*

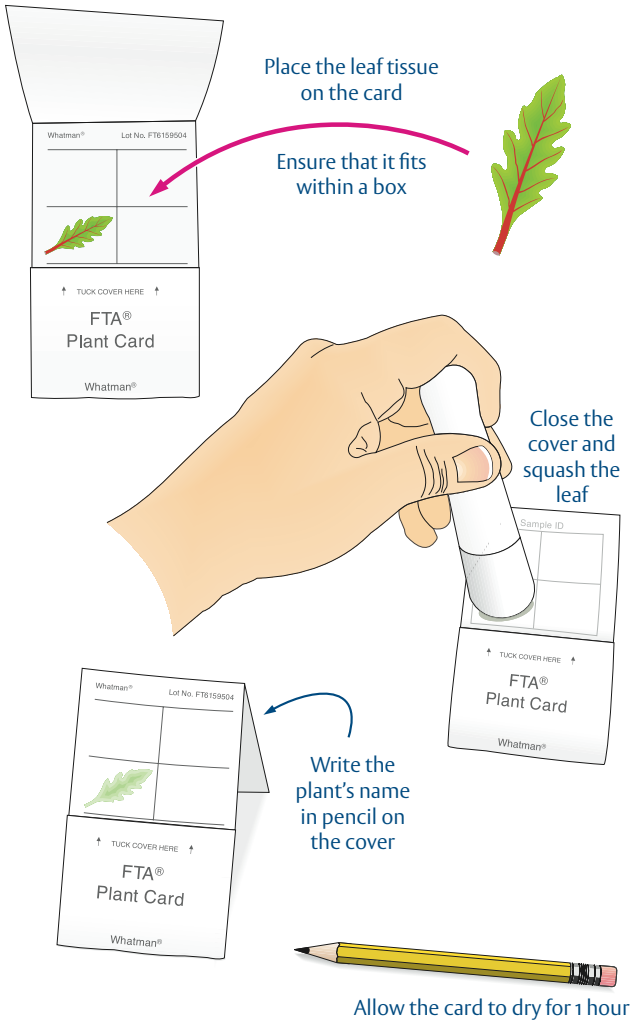
c. Throw away the plant material.

d. Write the plant's name in the appropriate 'Sample ID' box on the cover of the FTA® Plant Card. *The cover should be labelled **after** the plant material has been squashed onto the card, as the pestle can rub the writing off the front cover.*

e. With the cover folded back, allow the card to dry at room temperature *for a minimum of one hour*. **Important: Do not heat the card as this may fix inhibitors of the PCR onto the card.**

f. Once dry, the card can be stored indefinitely, sealed in a foil bag with a desiccant sachet at room temperature.

OPTIONAL STOPPING POINT



Notes

FTA® card is filter paper impregnated with SDS, Tris/EDTA (TE) buffer, and uric acid. SDS (a detergent) breaks open the plant cells. EDTA binds to metal ions that are required as co-factors by the enzymes that degrade DNA, so that the enzymes cannot work. Tris (a weak base) is needed to ensure the chelating action of the EDTA. The uric acid protects the DNA from degradation by free radicals.

2 Cleaning the DNA sample

a. Place a cutting board between the dry FTA® Plant Card and the backing. Place the tip of the punch over the area to be sampled *i.e.*, an area coloured green by chlorophyll. Press down firmly on the punch and rotate it to remove a paper disc. *To avoid cross-contamination, do not take a disc close to the edge of the box if the extracted sample has overlapped with another sample in an adjacent box.*

b. Transfer the small disc directly into a clear, colourless 1.5 mL microcentrifuge tube by pushing it out of the punch with the plastic 'wire' provided. Label the tube to indicate the species used. **Important: If the punch is to be used for a second sample, first clean the metal cutter by removing a disc of paper from an area of the card that does not have plant material applied to it. Discard this disc. This process should prevent contamination of subsequent samples.**

c. Using a 1 mL syringe fitted with a graduated yellow tip, add 100 µL of Purification Reagent to the tube containing the paper disc. ***Do not draw liquid into the syringe body.***

d. Close the tube and flick it several times to wash the disc. Flick every 30 seconds for 2 minutes. *Purification Reagent contains a detergent, which helps to wash cell debris, including PCR inhibitors, from the disc.* **Important: If the disc is not washed thoroughly the PCR may fail.**

e. Using the same yellow tip as in Step c, remove the Purification Reagent from the tube. Try to remove as much of the froth from the tube as you can, *but do not draw liquid or froth into the body of the syringe.*

f. Wash the disc again by repeating Steps c to e, using the same yellow tip for all operations.

g. Now, use a new yellow tip attached to the 1 mL syringe. Add 100 µL TE-1 buffer to the tube. *TE-1 is very dilute Tris-EDTA buffer.*

h. Close the tube and flick it to wash the disc. Flick every 30 seconds for 2 minutes. *This removes detergent that was in the Purification Reagent from the FTA® disc.*

i. Using the same yellow tip, remove and discard the TE-1 buffer from the tube.

j. Wash the disc again by repeating Steps g to i, using the same yellow tip.

k. Go on to conduct the PCR, or …

l. … alternatively, the disc can be dried in the tube by warming to ~50 °C. *You can use a hair-drier, radiator or incubator to speed up this process, but do not 'cook' the DNA excessively.* Once the disc is dry, place the closed tube in a sealed foil bag with a desiccant sachet and store it at room temperature until it is needed.

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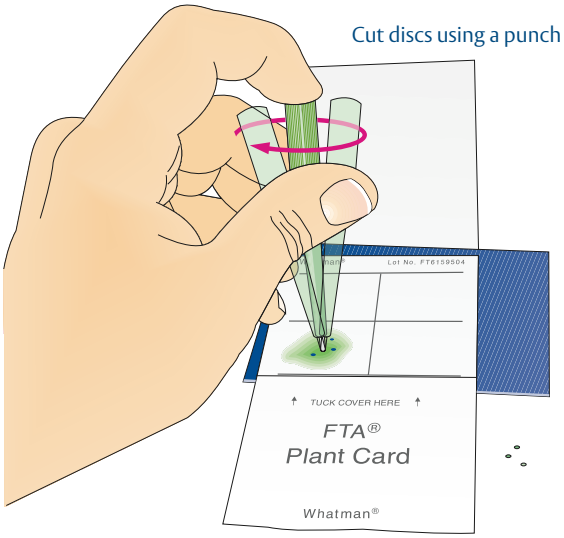
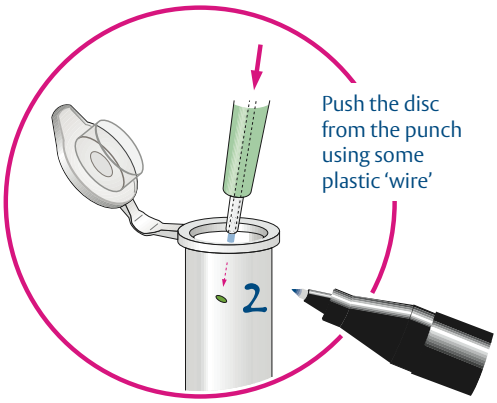
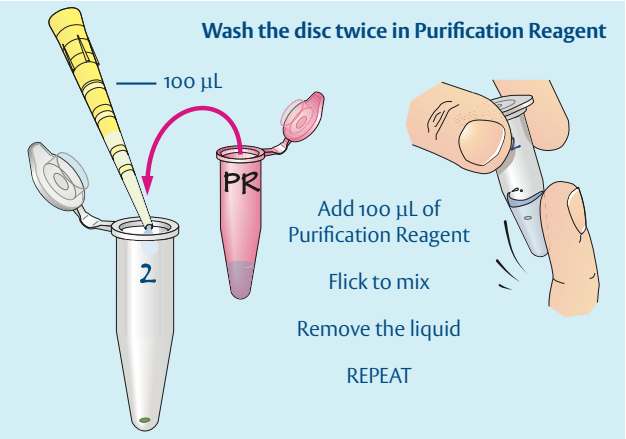
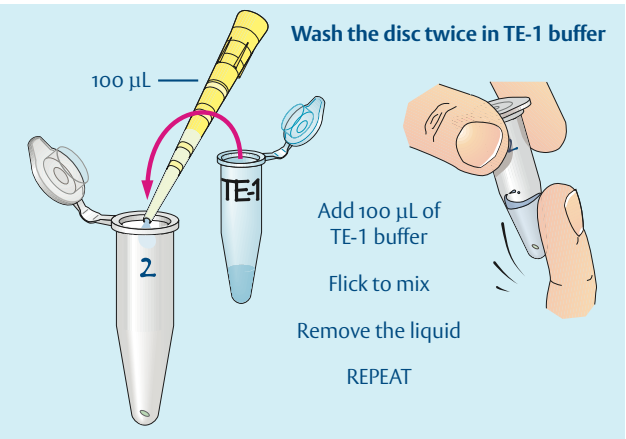








Testing for contamination

Because the PCR is so sensitive, it is important to check whether contamination with unwanted plant DNA has occurred. In addition to the disc for the plant species, take a disc from an area of the card that does not deliberately have plant material applied to it. Treat this disc in the same way as the 'plant' disc, washing it and so on as though it were a disc impregnated with plant material. This test will tell you whether unwanted DNA has contaminated the punch and samples taken with it. *Ideally, each person who sets up a PCR should do this test, but because the reagents are costly, only one test is usually run per class or group.*

Ideally, you should also test whether any of the PCR reagents are contaminated with DNA. This can be done by setting up a reaction with the two primers and water but with no paper disc. *It can be assumed, however, that all of the reagents supplied are free from contamination.*

Picomoles

The amount of DNA in the primer solutions is measured in picomoles (pmol). One picomole = 10^{-12} moles. A solution containing one picomole of DNA will contain 602,214,150,000 molecules of DNA.

3 Amplifying the chloroplast DNA

a. Using a microsyringe fitted with an unused, white, graduated tip for each reagent, add the following to a 0.5 mL microcentrifuge tube containing a PCR bead:

   - 10 µL of Primer 1 (containing 5 pmol of DNA per µL)
   - 10 µL of Primer 2 (containing 5 pmol of DNA per µL)
   - 4 µL of distilled water (add 6 µL if a *dry* FTA® disc is used)

   Ensure that the tip does not touch the PCR bead. If it does, the bead may stick to the tip. Instead, place the tip against the side of the tube above the bead. This will leave a drop of liquid on the side of the tube. The liquid will then run down to the bottom of the tube, causing the PCR bead to dissolve.

b. Close the tube. Flick the bottom of the tube gently to mix the contents and to help the PCR bead dissolve.

c. Spin the tube briefly in a microcentrifuge or tap the tube firmly on the bench to collect the liquid at the bottom of the tube.

d. Using a pair of forceps, place the FTA® disc prepared in Part 2 (above) in the tube containing the PCR reagents. **Important: Ensure that the disc is submerged in the liquid in the tube.**

e. Close the tube and label the top with the name of the plant used and your initials.

f. Place the tube in a foam floater, along with any tubes from others in your group or class.

g. To conduct the PCR, transfer the floater between three water baths as follows:

   94 °C for 2 minutes
   (ensures that the template DNA is single-stranded)

   94 °C for 30 seconds
   55 °C for 30 seconds
   72 °C for 30 seconds
   } Repeat this cycle 30 times

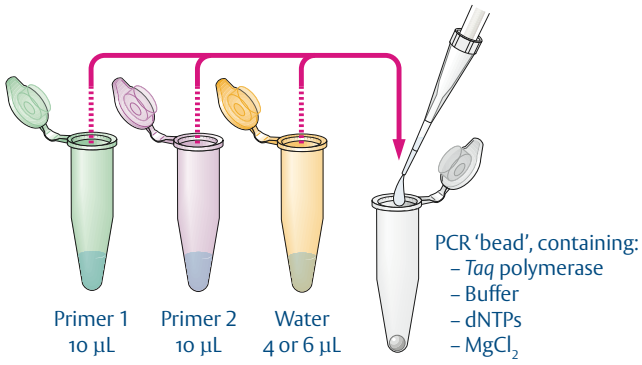
   72 °C for 2 minutes
   (ensures that the DNA fragments are fully extended).

   To maintain 94 °C it may be necessary to place a lid on the water bath when it is not in use. Take care not to scald yourself!

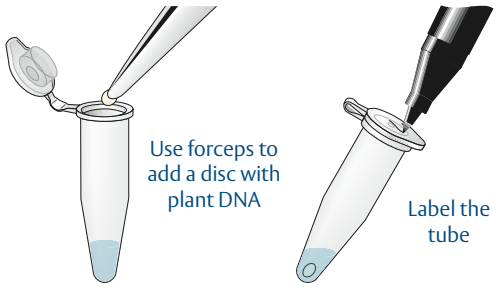
h. Go on to conduct the gel electrophoresis or …

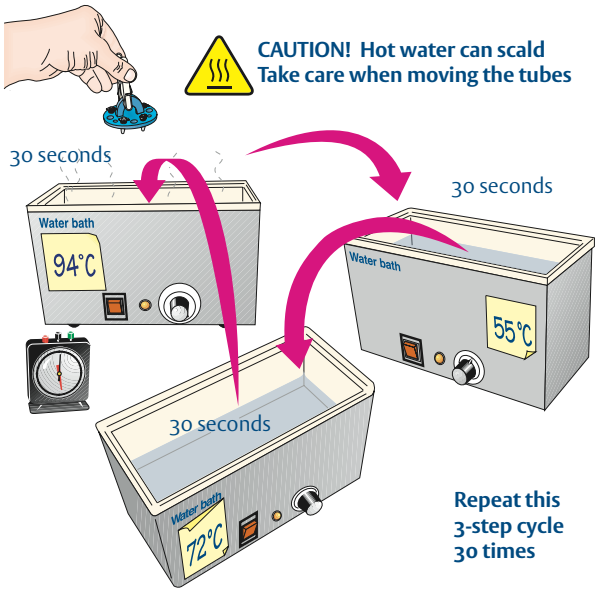
i. … alternatively, store the amplified DNA solutions in a freezer at –20 °C until they are required.

OPTIONAL STOPPING POINT



Primer 1: 5'–CGAAATCGGTAGACGCTACG–3'
Primer 2: 5'–GGGGATAGAGGGACTTGAAC–3'





4 DNA gel electrophoresis

I. Preparing the agarose gel

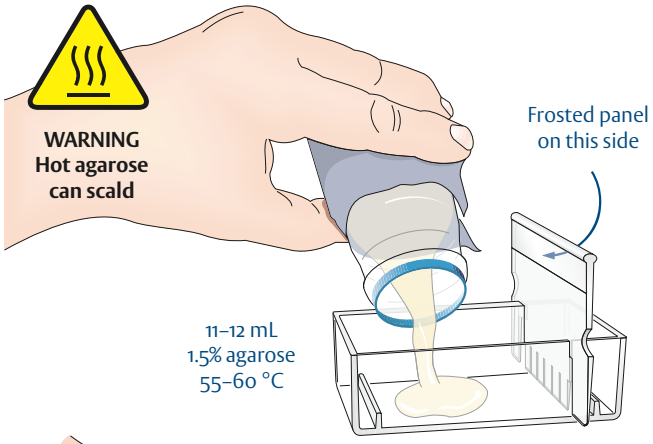
a. Use a microwave oven or a boiling water bath to melt some agarose gel (1.5% made up in TBE buffer). *Ensure that no lumps or fibres remain in the molten agarose — you can see these by holding the bottle up to the light.* Once it has melted, stand the molten agarose in a sealed container in water bath at 55–60 °C until it is needed.

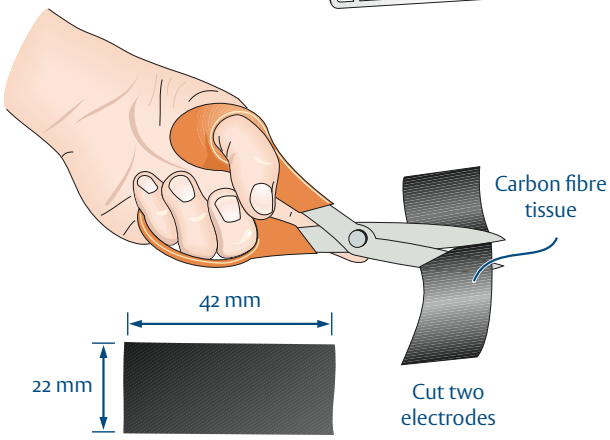
b. Place the electrophoresis tank on a *level* surface, where you can leave it undisturbed for the next 20–30 minutes. *This is necessary because if the gel sets at an angle, the DNA fragments will not run evenly through the gel.* Slot a 6-toothed comb in place at one end of the tank. Ensure that the frosted panel faces the near end of the tank.

c. Pour ~12 mL of molten agarose into the tank so that it fills the central cavity and flows under and between the teeth of the comb. Add just enough agarose so that the liquid is level with the top of the two ridges in the tank, and does not bow upwards. If you accidentally spill liquid into the areas outside the two ridges, do not worry — you can remove the agarose later, when it has set.

d. Leave the tank undisturbed until the liquid has set. *Agarose is opaque and looks slightly milky when the gel is set.*

e. While the gel is setting, cut two pieces of carbon fibre tissue, each about 42 mm x 22 mm. These will be the electrodes at either end of the electrophoresis tank.





II. Loading the gel

a. Pour slightly more than 10 mL of TBE buffer solution into the electrophoresis tank. The liquid should cover the surface of the gel to a depth of 2–3 mm and flood into the areas that will hold the electrodes.

b. *Very gently* ease the comb from the gel. As you do so, the buffer solution will fill the wells that formed. *Take care not to damage the wells as you remove the comb.*

c. Put the tank where you are going to 'run' it, where it will remain undisturbed.

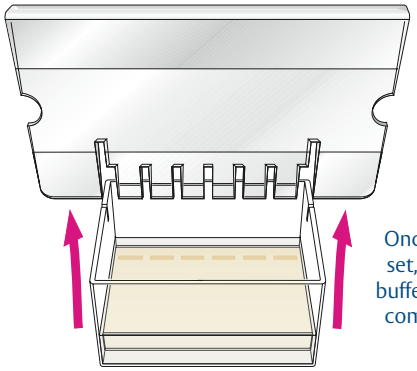
d. It is easier to see what you are doing next if the tank is placed on a dark surface, such as a piece of black card. Alternatively, a strip of black tape can be stuck onto the bottom of the tank beneath the wells. Put a clean white tip on the microsyringe. Add 2 µL of bromophenol blue loading dye to the tube containing the DNA you wish to load. Mix the dye into the DNA sample thoroughly, by drawing the mixture up and down in the microsyringe. Pipette about 10 µL of the loading dye and DNA mixture into one of the wells, holding the tip above the well but under the buffer solution (see picture). *Take great care not to puncture the bottom of the well with the microsyringe tip.*

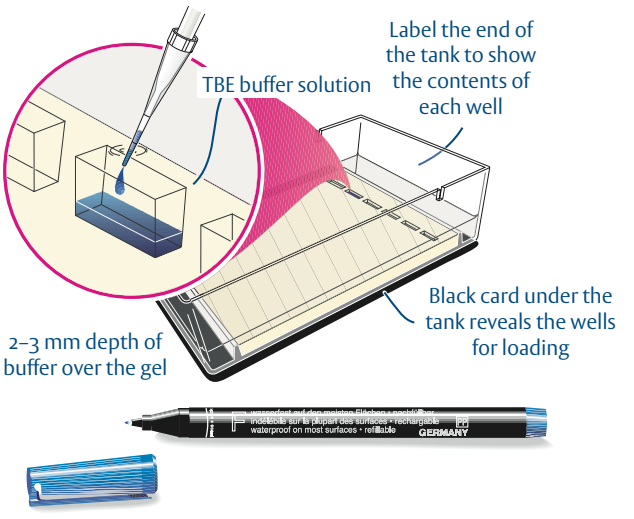
e. Make a note of which DNA you have put into the well (*e.g.*, write on the side of the tank with a marker pen).

f. Repeat Steps d and e with each DNA sample. *Remember to use a new tip for each sample, to avoid cross-contamination.*

g. Load 10 µL of diluted DNA 'ruler' into one of the wells.

h. On one gel in the class, also load 10 µL of the test sample that had no plant DNA added to the reaction mixture.





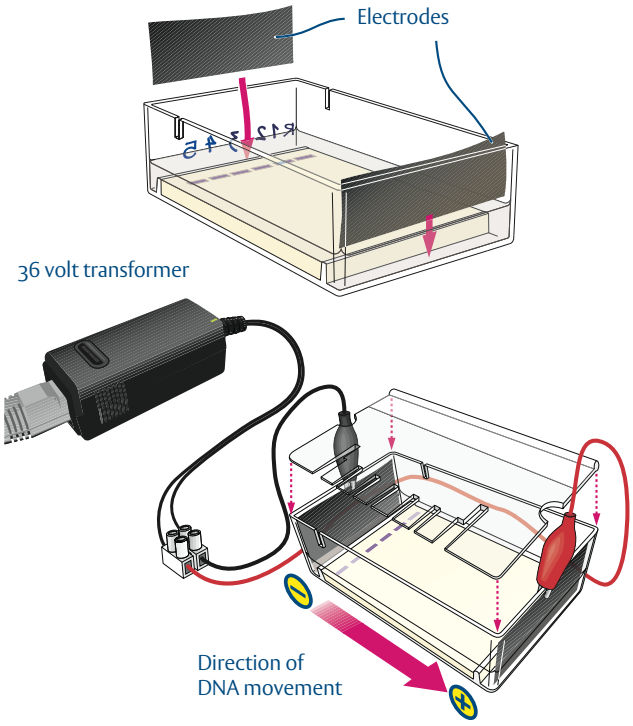
III. 'Running' the gel

a. Fit one electrode at each end of the tank as shown in the illustration. Join the electrodes to the power supply using wires with crocodile clips. An NCBE mains transformer or sufficient 9 V batteries (in series) should be used to give a total voltage of **no more than 36 volts**. *Ensure that the positive terminal of the power supply is connected to the electrode* ***furthest*** *from the wells.*

b. Check that contact is made between the buffer solution and the electrodes (with care, you can add more buffer if necessary). Place the comb over the tank to reduce evaporation during the electrophoresis. Leave the gel to 'run', undisturbed. At 36 volts, it will take 3½ hours to run a gel of 1.5% agarose.

c. Disconnect the power supply once the blue loading dye is within 10 mm of the end of the gel. *The DNA fragments (~400 base pairs in length) will be just behind the loading dye.* **Important: If you leave the power on, the DNA will run off the end of the gel and be lost!**

d. Rinse the crocodile clips in tap water and dry them thoroughly to prevent corrosion.



Notes

Ions in the TBE buffer used in and above the gel conduct electricity. This buffer is alkaline. Under alkaline conditions the phosphate groups of the DNA are negatively-charged and therefore the DNA moves towards the positive electrode (anode) when a current is applied. EDTA in the buffer 'mops up' or chelates divalent cations. This helps to prevent damage to the DNA as such ions are required as co-factors by DNA-degrading enzymes.

APPLY NO MORE THAN 36 VOLTS!

Serious or lethal electrical shock may occur if you connect the equipment directly to a mains electricity supply.

IV. Staining the DNA

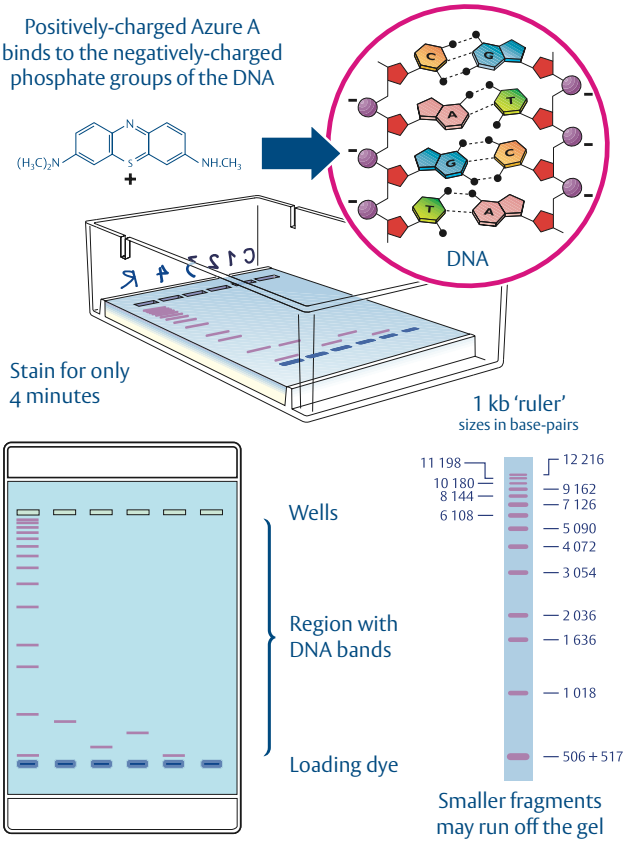
a. Remove and dispose of the electrodes. Pour off the buffer solution. The buffer may be retained and re-used several times, but it will not last indefinitely.

b. Put on some plastic gloves to prevent the stain from touching your skin.

c. Pour about 10 mL of the stain solution (0.04% Azure A in 20% ethanol) onto the surface of the gel.

d. Leave it for *exactly* 4 minutes, then return the stain to a bottle for re-use. (Like the buffer, the DNA stain can be re-used several times before you need to replace it. You may find, however, that older stain needs to be left on the gel for a little longer than 4 minutes.)

e. Rinse the surface of the gel with water 3 or 4 times, *but do not leave any water on the gel, as this will remove stain from the top of the gel.* The remaining stain will gradually move down through the gel, staining the DNA as it does so. Faint bands should start to appear after 10 minutes. The best results will be seen if the gel is left to 'develop' overnight. Put the tank in a plastic bag if you do this, to prevent the gel from drying out.



Notes

It is not necessary to destain the gel if you follow this procedure.

Stained gels may be stored indefinitely in a sealed plastic bag, kept in a dark, cool place such as a refrigerator. Exposure to bright light will cause the bands on the gel to fade.

Safety guidelines

FTA® cards and PCR reagents

FTA® paper is non-toxic to humans and hypo-allergenic. The PCR reagents are also non-toxic. Cleanliness is important to prevent cross-contamination and ensure success, so dirty tubes and tips should not be re-used. Used plastic tubes and tips, which are made of polypropylene, can be disposed of in the normal waste.

Agarose gel

If a microwave oven is used to melt the agarose gel, ensure that the gel is placed in an *unsealed* container. A boiling water bath or hotplate may be used instead, but the gel must be swirled as it melts to prevent charring. The use of a Bunsen burner to melt agarose is *not* recommended.

Molten agarose can scald and it must be handled with care, especially just after it has been heated in a microwave oven.

Hot water baths

If you use water baths for the PCR (rather than an automated thermal cycler), take care not to scald yourself when transferring tubes between them. Use forceps or a clothes peg to hold the foam floater and/or wear heat-resistant gloves to protect your hands from the hot water and steam.

TBE buffer (Tris-Borate-EDTA)

When used as directed, this buffer presents no serious safety hazards. Spent buffer can be washed down the drain.

Loading dye (Bromophenol blue)

When used as directed, this loading dye presents no hazard. Used loading dye can be washed down the drain.

Electrode tissue

The carbon fibre electrode tissue may release small fibres, which can cause skin irritation if you handle the tissue a lot. Wear protective gloves if you find the tissue unpleasant to handle. The fibres are too large to enter the lungs however, so it is not necessary to wear a face mask. The fibres are soluble in body fluids and are completely biodegradable.

Electrical supply

The gel electrophoresis equipment was designed to be used with direct current at low voltages (≤36 volts) with dry cell batteries. ***Under no circumstances should this voltage be exceeded, as the live electrical components are exposed.***

Serious or lethal electrical shock may occur if you connect the equipment directly to a mains electricity supply.

DNA stain (Azure A)

The concentrated DNA stain solution is flammable and it must be kept away from naked flames. The stain is Azure A, which when diluted as directed, forms a 0.04% solution in 20% ethanol. At this concentration it presents no serious safety hazard, although care should be taken to prevent splashes on the skin or eyes *e.g.*, wear protective gloves and glasses. Used stain may be washed down the drain.

Further information

Printed publications

Mullis, K.B. (1990) The unusual origin of the polymerase chain reaction. *Scientific American* **262**, 36–43.

Making PCR. A story of biotechnology by Paul Rabinow (1996) The University of Chicago Press. ISBN: 0 226 70147 6.

Willmott, C. (1998) An introduction to the polymerase chain reaction. *School Science Review* **80**, 49–54.

Taberlet, P. *et al.* (1991) Universal primers for the amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17**, 1105–1109.

Streicher, H. and Brodte, A. (2002) Introducing students to DNA: Identifying nutritional plants in a simple tried and tested laboratory experiment. *Biochemical Education* **30**, 104–105.

Molecular structure data

The molecular images in this booklet were created using data from the *Protein Data Bank*: **www.rcsb.org/pdb**

The data for *Taq* polymerase was published in: Eom, S.H., *et al.* (1996) Structure of *Taq* polymerase with DNA at the polymerase active site. *Nature* **382**, 278–281. [PDB ID: 1TAU].

The Rubisco molecule is from: Taylor, T.C., and Andersson, I. (1997) The structure of the complex between rubisco and its natural substrate ribulose 1,5-bisphosphate. *Journal of Molecular Biology* **265**, 432–444. [PDB ID: 1RCX].

The software used to produce the images was *VMD* (Visual Molecular Dynamics). This software can be downloaded free-of-charge for Macintosh, Windows, Linux and other platforms from: **www.ks.uiuc.edu/Research/vmd/**

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