



The PCR and Plant evolution

1.4 Teacher's notes

Equipment and materials

Equipment and materials

Required by each student or working group
(there is sufficient material in the kit for 16 amplifications)

Equipment included in the kit

- Punch for cutting 2 mm discs
- Pipettes to measure 2, 10, and 100 μL volumes
- Floating microcentrifuge tube holder

Equipment not included in the kit

- Pestle
- Pair of forceps
- DNA electrophoresis equipment and power supply
- Microsyringe dispensing units
- Protective gloves (to protect hands from DNA stain)
- Pencil
- Permanent marker pen for marking the tubes and the lanes on the gel tank
- Access to three water baths maintained at 55, 72 and 94 $^{\circ}\text{C}$, or a thermal cycler (PCR machine)

Materials included in the kit

- Microcentrifuge tubes (1.5 mL)
- Micropipette tips
- Whatman[®] FTA[®] Plant Card
- FTA[®] Purification Reagent (~ 350 μL per plant sample)
- TE-1 buffer (~ 350 μL per plant sample)
- 'Ready-to-go' PCR beads (1 per plant sample)
- PCR primers and sterile distilled water, for each sample as follows:
 - 10 μL of Primer 1
 - 10 μL of Primer 2
 - ~ 6 μL of sterile distilled water
- 1 kb DNA ladder/ruler (only one is required per gel)
- Bromophenol blue loading dye (~2 μL per sample)
- Electrophoresis (TBE) buffer solution
- Agarose powder (make a 1.5% agarose gel by dissolving the powder in diluted electrophoresis buffer)
- Toluidine blue O (0.04% w/v) in water (for staining DNA)

Dispensing the reagents

For students to use

Purification reagent

6.5 mL of the Purification reagent is supplied. Each student or group of students will need 350 μL of this solution, dispensed into a red, 1.5 mL microcentrifuge tube.

TE-1 buffer

6.5 mL of the TE-1 buffer is supplied. Each student or group of students will need 350 μL of this solution, dispensed into a blue, 1.5 mL microcentrifuge tube.

PCR primers and water

Use the 20 μL *Volac* pipette provided and clean tips to dispense 20 μL of each of Primer 1, Primer 2 and sterile distilled water for each student or group of students. This is more than they need, but these reagents are cheap.

Primer 1 should go in a green 0.5 mL microcentrifuge tube, Primer 2 into a violet tube, and the water into an orange tube. The 'stock' solutions are provided in screw-capped tubes with lids of the same colours.

DNA ruler/ladder (1 kb)

130 μL of ready-diluted DNA ruler is provided in a 1.5 mL screw-capped tube. Before use, add 12 μL of bromophenol blue loading dye (use a microsyringe unit with a white graduated tip). Mix well by drawing the liquid up and down in the tip, then dispense 14 μL into each of 8 yellow 0.5 mL microcentrifuge tubes for students to use.

These diluted solutions can be stored in a freezer for up to a month before use: the undiluted stock can be stored indefinitely in a freezer.

IMPORTANT: Please note that the DNA 'ladder' or 'ruler' supplied in the kit is NOT the same as that described in the accompanying Student's guide. It is an improved version with more regularly-distributed DNA fragment sizes (see sheet provided with this guide).

Additional technical details

'Ready-to-go' PCR beads

Each 'Ready-to-go' PCR bead is designed for a single 25 μL reaction. On resuspending to 25 μL , the final component concentrations are:

Tris-HCl (pH 9.0)	20 mM
KCl	50 mM
MgCl ₂	1.5 mM
dNTP mixture	0.2 mM of each dNTP
Taq polymerase	2.5 units

DNA extraction reagents

The Whatman[®] FTA[®] cards contain SDS (a detergent), TE buffer and other proprietary reagents. The Purification Reagent is also proprietary. The TE-1 is simply dilute Tris-EDTA buffer.

Primer sequences

The primers used in the protocol typically span a 400 bp fragment. Their sequences are:

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

Plants to test

We have tested and obtained good results with:

- Nasturtium
- Spinach
- Rocket
- Spring onion
- Watercress*
- *Scenedesmus quadricauda*
- *Chlorella vulgaris*
- Red chard
- Sweet and chilli peppers (skin of green varieties)
- Chive
- Tatsoi
- Corriander
- Mange tout
- Parsley
- Brussels sprout
- *Selenastrum gracile*
- Mizuna

* *Watercress consistently gives a larger PCR product.*

Species with tough or fibrous leaves do not work well with this extraction method. In our (limited) tests, *none* of the following plants gave reliable amplification:

- 'Cress' (*Brassica napus* or *Lepidium sativum* seedlings);
- Cabbage and broccoli (both plants are the same species, *Brassica oleracea*);
- Thyme, basil or mint;
- Cucumber or courgette skin;
- Weeping fig (*Ficus benjamina*).

Timing

This investigation is a complex multi-step process. Careful planning is therefore necessary to fit it into a normal series of school lessons or a single practical session. The whole procedure, including drying the FTA® cards and manual PCR but excluding the electrophoresis, takes about three hours. The duration of the electrophoresis depends upon the equipment and voltage used. As a rough guide, the timing of this protocol is as follows:

Extracting the DNA onto FTA® cards: 15 minutes

Drying time: 60 minutes

OPTIONAL STOP POINT

Cleaning the DNA samples: 20 minutes

OPTIONAL STOP POINT

Preparing the PCR reagents: 10 minutes

PCR manual cycling: 50 minutes

PCR thermal cycler: ~ 90 minutes

OPTIONAL STOP POINT

Preparing and loading the gel: 30 minutes

Electrophoresis: 30 minutes – 3½ hours

Staining the gel: 10 minutes

Additional resources

The polymerase chain reaction

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

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.

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

An animated simulation of the PCR can be downloaded from Cold Spring Harbor Laboratory's Dolan DNA Learning Center: www.dnalc.org/ddnalc/resources/animations.html

The Protein Data Bank (www.rcsb.org/pdb) features DNA polymerase in its 'Molecule of the month' series.

Plant classification

Guidance for selecting plant species is provided by the web-based *Tree of Life* project: <http://tolweb.org/>

The classification produced by the *Angiosperm Phylogeny Group* is described in:

APG (2003) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants; APG II. *Botanical Journal of the Linnean Society* **141**, 399–436. This paper and additional information is available on-line from: www.mobot.org/MOBOT/research/APweb/

Molecular structures

The molecular images in the student's booklet were created using structure data from the *Protein Data Bank*. The software used to produce the images was *Visual Molecular Dynamics*. VMD can be downloaded free-of-charge for Macintosh, Windows, Linux and other platforms from: www.ks.uiuc.edu/Research/vmd/

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 (Toluidine blue O)

The concentrated DNA stain solution is Toluidine blue O, which when diluted as directed, forms a 0.04% solution in water. 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. [This stain replaces Azure A, which was previously supplied with this kit.]

Acknowledgements

Science and Plants for Schools

Several people contributed to the development of the original practical protocol from which this kit was developed and we wish to thank them all.

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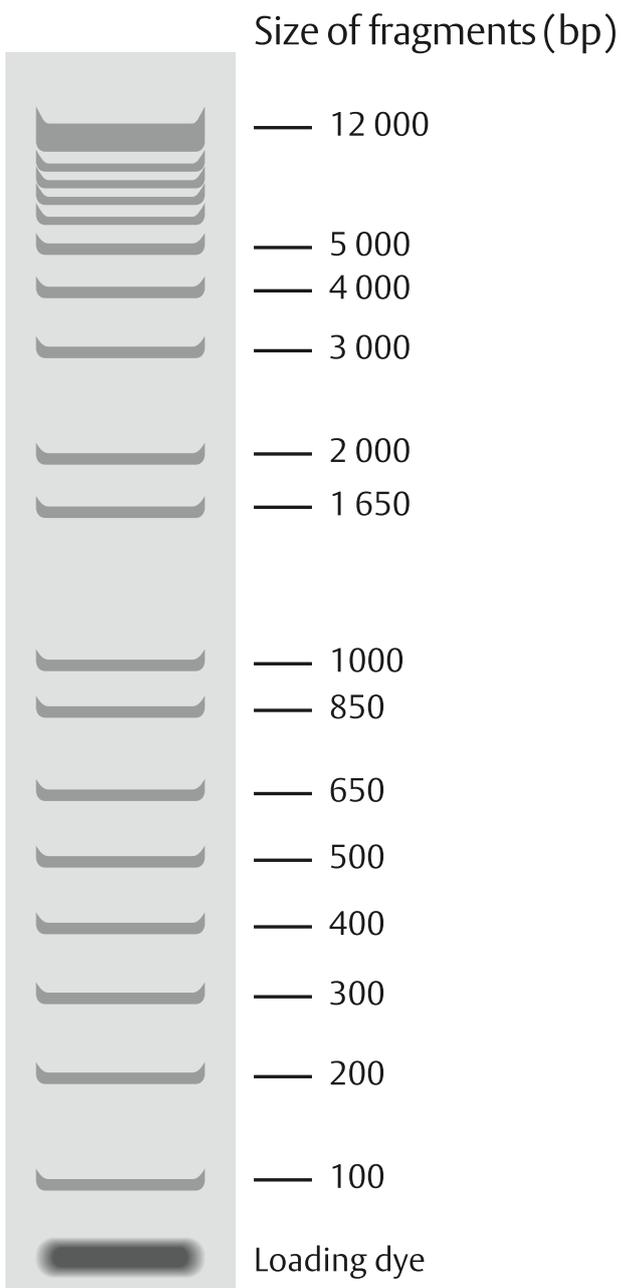
Paul Beaumont, then Director of SAPS, kindly read through the draft version of the Student's guide and made many corrections.

University of Reading

Andy Harrison at the *National Centre for Biotechnology Education* adapted the original SAPS protocol. He developed a simpler and cheaper method of extracting plant DNA and a revised PCR protocol. Andy also did most of the work in converting a laboratory procedure into kit form.

Julie Hawkins in the *School of Biological Sciences* kindly advised on the section in the Student's booklet dealing with modern plant classification.

New DNA ladder



A DNA 'ladder' or 'ruler' consisting of fragments up to 1.2 kb is included so that the DNA under investigation can be compared with fragments of known sizes.

The fragment sizes are given in base pairs (bp).

The bromophenol blue loading dye moves through the gel as if it were made of 500 bp DNA fragments.

With a six-tooth comb, you should use 2 μg of 'ruler' solution per well. With the 4-tooth comb you will need about 3 μg per well.

Note: This DNA ruler is NOT the same as the one shown in the student's guide. It is an improved version with more regularly-distributed DNA fragment sizes.