Practical

Amplification of chloroplast DNA using the polymerase chain reaction (PCR): a practical activity for secondary school students

Kenny Hamilton[1], Jan Barfoot[2], Kathleen E Crawford[3*], Craig G Simpson[4], Paul C Beaumont[5] and Mary Bownes[2].

[1]Breadalbane Academy, Aberfeldy, [2]Scottish Initiative for Biotechnology Education, Dunfermline, [3]SAPS Biotechnology Scotland Project, Edinburgh, [4]Scottish Crop Research Institute, Invergowrie, and [5]Science and Plants for Schools, Homerton College, Cambridge

We describe a polymerase chain reaction (PCR) protocol suitable for use in secondary schools and colleges. This PCR protocol can be used to investigate genetic variation between plants. The protocol makes use of primers which are complementary to sequences of nucleotides that are highly conserved across different plant genera. The regions of chloroplast DNA amplified lie between these conserved sequences and are non-coding. These non-coding regions display a high frequency of mutations and show relatively high rates of evolutionary change. Thus it is possible to use the protocol to explore evolutionary relationships between plants. Results from *Brassica oleracea* can be used to highlight genetic similarity and differences within and across genera. The protocol is robust and is suitable for use either with a thermocycler or a series of water-baths, thus making it accessible for use in most schools and colleges.
Key words: PCR; Polymerase chain reaction; Chloroplast; Practical activity; Evolutionary relationships

Introduction

The polymerase chain reaction (PCR) is an important molecular technique with applications in virtually all biological, biomedical and biotechnological areas of science (Gillaspy, 2004; Micklos and Freyer, 2003; Smith, 1996; Walker and Rapley, 2000). PCR produces many copies of a specific section of DNA and, therefore, allows that DNA fragment to be manipulated and analysed in greater detail. PCR has been described as the genetic equivalent of finding the needle in a haystack and then producing a haystack of needles (Mullis, 1990). The teaching of PCR can also be used to reinforce important aspects of basic cell biology. For example, PCR involves the separation of double-stranded DNA and the extension of complementary strands by a DNA polymerase. It is, in effect, an *in vitro* version of DNA synthesis. Furthermore, the understanding of basic concepts such as the structure of DNA, complementary base pair binding, hydrogen and covalent bonding can be strengthened through discussion of PCR.

In our experience, many students find the underpinning theory of PCR difficult to understand, despite the existence of many animations and diagrams to aid the teaching of PCR (for example, *www.dnalc.org/ddnalc/resources/pcr.html* – accessed July 2006). In a review of research into the role of practical work in the classroom (Harlen and Wake, 1999), it was concluded that appropriate, well thought out, practical work (in terms of purpose and learning objectives) can provide students with first-hand experience of an aspect of science

and allow them to formulate a 'theory' and test ideas by making predictions. Finally, practical PCR can provide pupils with a valuable experience of 'doing science' rather than 'learning science' (Harlen and Wake, 1999). Thus, a PCR practical will not only demonstrate the technique but also lead to increased understanding of DNA technology and its applications.

The molecular concepts behind, and applications of, PCR have been part of the Scottish Advanced Higher Biology curriculum for six years (see Table 2 for a fuller list of the various Curriculum Authorities and their associated subject specifications). At present, and as a result of our own interaction with teachers, we have found that little practical work involving PCR is carried out in schools in the UK. This lack of practical work results from two principal causes:

1. To date, there is a lack of a robust, simple protocol which is appropriate for use in schools. A protocol is required that (i) uses accessible reagents, which do not present significant hazards for use in classrooms, (ii) uses reagents which are easy to store, (iii) is suitable for use within the constraints of school timetables (e.g. within single and/or double teaching periods), (iv) uses DNA which can be easily obtained.

2. A typical PCR protocol usually involves the use of a thermocycler; such devices are normally beyond the budget of science departments in schools. Before thermocyclers were available, PCR protocols often involved

Table 1. Plants for which this protocol is known to work

Common name	Latin name	Common name	Latin Name	Common Name	Latin Name
Pak Choi	Brassica rapa subsp. Chinensis	Celery leaf	Apium graveolens	Brussels Sprout	Brassica oleracea var. gemmifera
Coriander	Coriandrum sativum	Cress	Lepidium sativum	Curly Kale	Brassica oleracea var. acephela
Salad Rocket	Eruca vesicaria	Iceberg Lettuce	Lactuca sativa	Green Cabbage	Brassica oleracea var. capitata
Savoy Cabbage	Brassica oleracea var. capitata	Broccoli	Brassica oleracea var. italica	Flat Leaf Parsley	Petroselinum crispum
Spinach	Spinacia oleracea	Red Chard	Beta vulgaris	Spring Onion	Allium cepa
Spider Plant	Chlorophytum comosum	Cauliflower leaf	Brassica oleracea, var. botrytis		

the use of a series of water-baths or heating blocks set at different temperatures. For use in a school, a suitable PCR protocol needs to be effective for use with either thermocycler or water-bath systems. While such a protocol may involve less stringent conditions of temperature and reaction times than others used for research or commercial purposes, it still needs to work effectively.

Due to the importance of PCR as a molecular technique and the lack of an appropriate practical activity to support the teaching of PCR, we have developed a protocol suitable for use within schools. This protocol allows the robust and efficient amplification of sections of easily obtainable chloroplast DNA from both *Brassica* and non-*Brassica* plant species and thereby allows students to explore the differences and evolutionary relationships between plants.
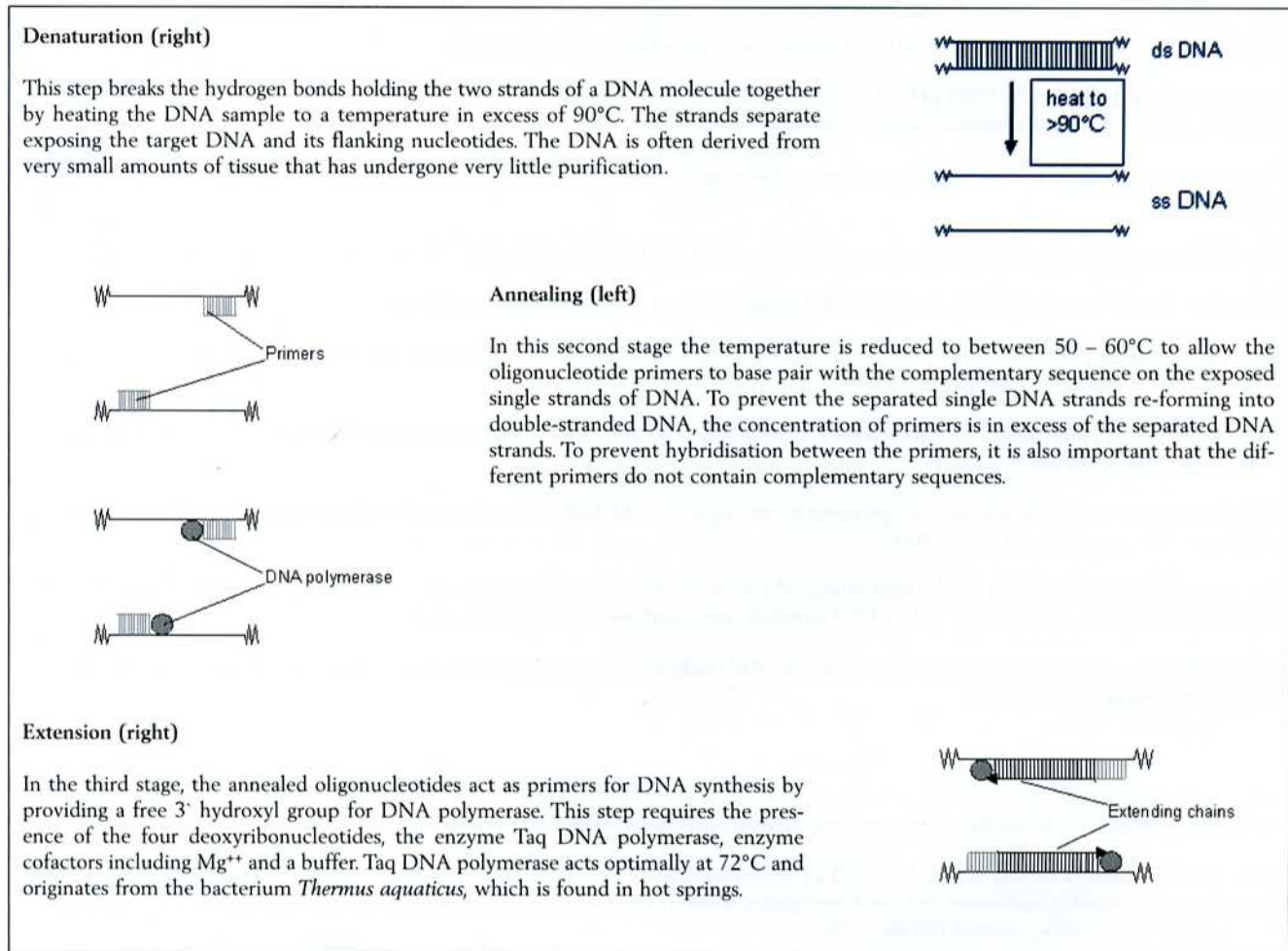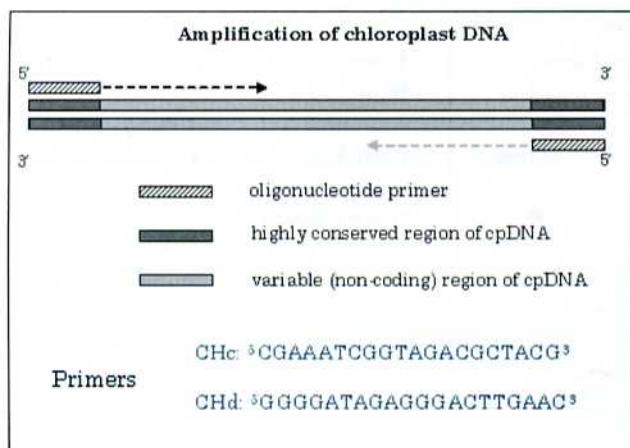
PCR – the technique

PCR is a technique which enables a specific region of DNA to be amplified provided the nucleotide sequences at each end of the region are known. The process of PCR amplification involves three major steps:

1. **Denaturation** of DNA to separate DNA strands.
2. **Annealing** of primers. Primers are synthetic oligonucleotides (short pieces of single stranded DNA, usually 19-25 bases long). The sequence of bases complements the bases that flank each end of the target DNA.
3. **Extension** of new strands of DNA by Taq DNA polymerase.

Completion of these three stages (a cycle) leads to a doubling of the number of DNA molecules present. The cycle is repeated many times and the number of DNA molecules

Figure 1. Summary of the Polymerase Chain Reaction

Denaturation (right)

This step breaks the hydrogen bonds holding the two strands of a DNA molecule together by heating the DNA sample to a temperature in excess of 90°C. The strands separate exposing the target DNA and its flanking nucleotides. The DNA is often derived from very small amounts of tissue that has undergone very little purification.

Annealing (left)

In this second stage the temperature is reduced to between 50 – 60°C to allow the oligonucleotide primers to base pair with the complementary sequence on the exposed single strands of DNA. To prevent the separated single DNA strands re-forming into double-stranded DNA, the concentration of primers is in excess of the separated DNA strands. To prevent hybridisation between the primers, it is also important that the different primers do not contain complementary sequences.

Extension (right)

In the third stage, the annealed oligonucleotides act as primers for DNA synthesis by providing a free 3' hydroxyl group for DNA polymerase. This step requires the presence of the four deoxyribonucleotides, the enzyme Taq DNA polymerase, enzyme cofactors including Mg^{++} and a buffer. Taq DNA polymerase acts optimally at 72°C and originates from the bacterium *Thermus aquaticus*, which is found in hot springs.

Figure 2. *Amplification of chloroplast DNA.* The block dark grey areas represent known nucleotide sequences that form part of a highly-conserved tRNA gene and flank a variable non-coding region of the target DNA (signified by the block grey area). The base sequences of the synthetic oligonucleotides, shown as diagonally striped boxes, complement those of the tRNA gene (block dark grey areas) and base pair to give DNA primed ready for DNA synthesis (Taberlet *et al*, 1991). The DNA sequence between the two oligonucleotide primers is synthesised by Taq DNA polymerase and amplified by successive rounds or cycles of denaturation, primer annealing and DNA synthesis.

increases exponentially. The three steps of PCR are explained in more detail in Figure 1. More detailed consideration of the amplification process is beyond the scope of this manuscript (for further information, see Micklos and Freyer, 2003). It should be noted that after 30 amplification cycles, there will

be 2^{28} molecules of DNA generated for each target DNA molecule present at the start.

In this practical, a section of chloroplast DNA (cpDNA) is amplified. Chloroplasts are plant cell organelles that are found in numbers of up to 50 in a single cell and contain their own self-replicating DNA (general DNA information and the DNA sequences of different plant chloroplasts can be found at: *http://evogen.jgi.doe.gov/second_levels/chloroplasts/-cpDNA_info.html* and the origins of eukaryotic chloroplasts can be studied at: *http://hypnea.botany.uwc.ac.za/phylogeny-/classif/euk1.htm* – accessed July 2006).

Chloroplast DNA is a circular molecule that typically ranges from 120-160 kilobase pairs in length in higher plants. In most higher plants, cpDNA is inherited maternally, undergoes limited recombination and has a highly conserved gene order (Soltis *et al*, 1992). The primers selected for this PCR protocol were specific to genes that code for chloroplast transfer RNAs (tRNA acts as an adaptor molecule in protein synthesis, matching amino acids to their appropriate codons) and have been widely used for phylogenetic analysis of plant chloroplasts (Taberlet *et al*, 1991). Chloroplast tRNA genes are highly conserved between plant species, so nucleotide sequences make ideal consensus or universal primers.

Whilst the primers are located in the highly conserved regions of the cpDNA molecule, the region of DNA amplified lies between or within the tRNA genes and consists of the more variable non-coding regions of cpDNA (see Figure 2). These regions display the highest frequency of mutations and show relatively high rates of evolutionary change. These

Box 1. Technical preparations and resources

Green plant material was collected and/or bought fresh from within house or a supermarket.

2% SDS Tris NaCl EDTA (2% TNE) pH 8.0 - 100 mM Tris HCl, 100 mM NaCl, 50 mM ethylenediaminetetraacetic acid disodium salt (EDTA*), and 2% SDS w/v. Chemicals were purchased from Sigma-Aldrich.

The **primers** were bought on-line from Sigma Genosys (*www.sigma-genosys.co.uk*) and made up to a final concentration of 20 µM in distilled water.

The **PCR reagents** were purchased in the form of a dried pellet ('PuRe Taq Ready-To-Go™ PCR Beads') from Amersham Biosciences.

TE buffer - 10 mM Tris HCl pH 7.5, 1 mM EDTA. Chemicals were purchased from Sigma-Aldrich.

Cresol Red Loading Dye - 28% sucrose and 0.02% Cresol Red (Sigma-Aldrich) was made up in Tris EDTA (TE) buffer. Any other suitable DNA sample loading dye can be used.

Tris borate EDTA (TBE) buffer - 45 mM Tris base, 45 mM boric acid and 2 mM EDTA disodium salt. pH to 8.0. Chemicals were purchased from Sigma-Aldrich.

1.5% agarose gels for electrophoresis were prepared by mixing in 1 x TBE buffer. The mix was heated by microwave to melt the agarose and poured into an agarose gel well former.

Tris acetate EDTA (TAE) buffer can be used instead of TBE buffer for this practical. TAE buffer - 40 mM Tris base, 20 mM glacial acetic acid and 1 mM EDTA disodium salt. pH to 8.0. Chemicals were purchased from Sigma-Aldrich.

DNA Stains - Fast Blast DNA Stain (Bio-Rad - *www.Bio-Rad.co.uk*) or Azure A (National Centre for Biotechnology Education, NCBE - *www.ncbe.reading.ac.uk*) were used.

Please note many of the solutions listed above can be purchased pre-prepared from NBCE or Bio-Rad.

Other materials needed include microtubes (NCBE), micropipettes (NCBE), Pasteur pipettes, plastic homogeniser (Sigma-Aldrich, 'Pellet Pestles') or other suitable instrument, industrial methylated spirits (IMS) cooled to -20°C, distilled water, microcentrifuge (NCBE or Bio-Rad) and electrophoresis equipment (NCBE or Bio-Rad).