

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

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

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.

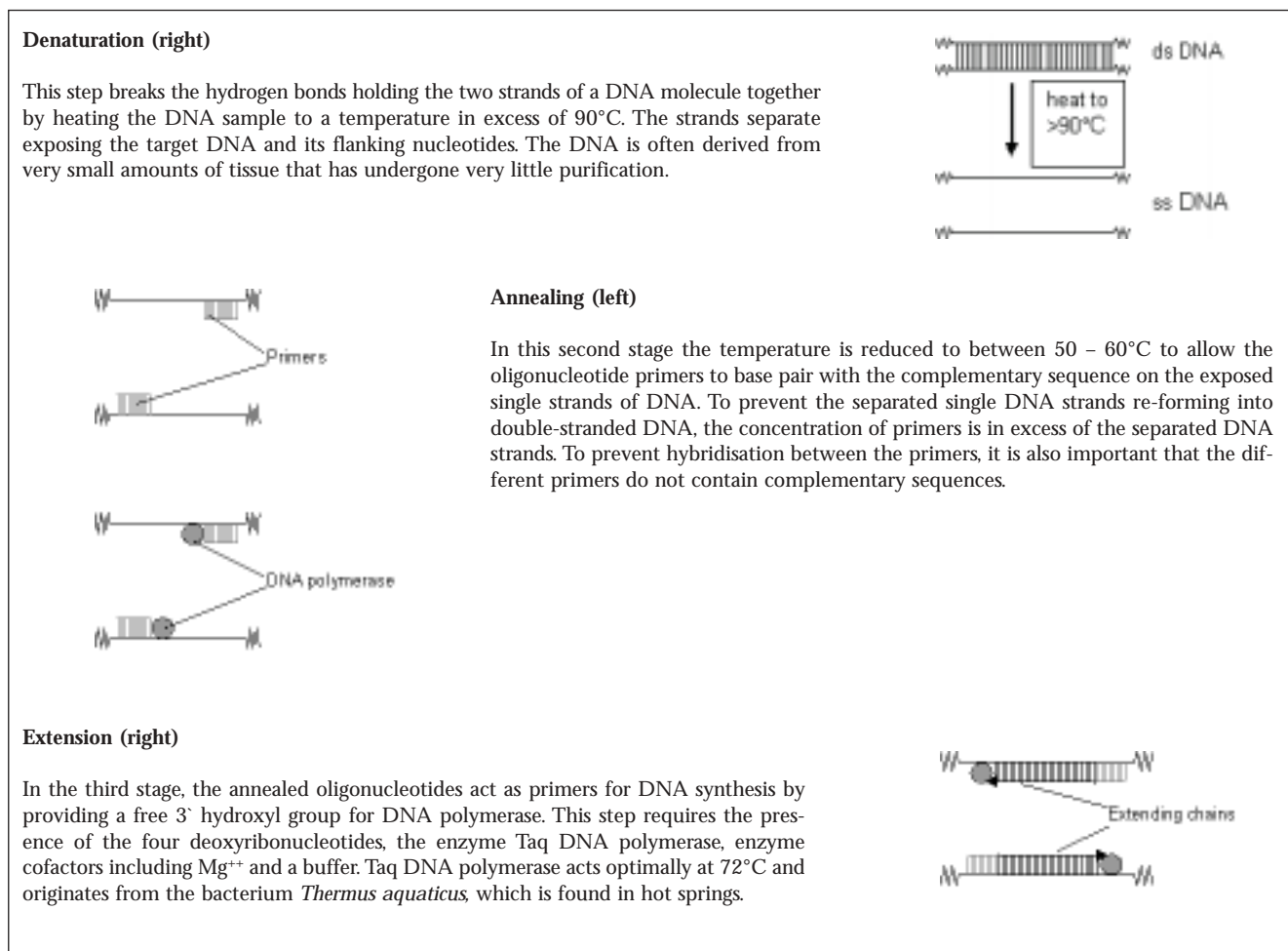
PCR – the technique

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



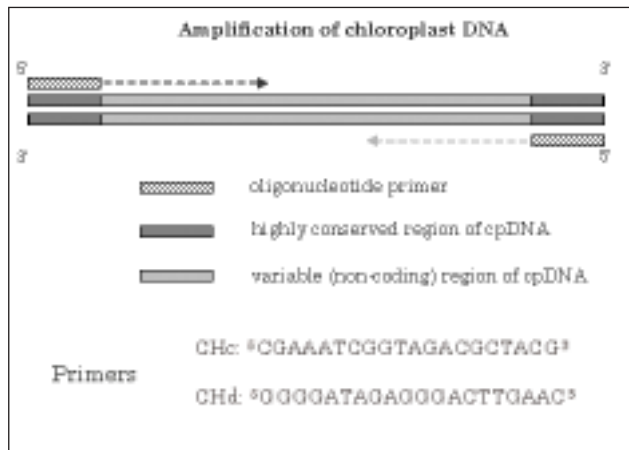


Figure 2. Amplification of chloroplast DNA. The block dark grey areas represent known nucleotide sequences that form part of a highly-conserved tRNA gene and flank a variable non-coding region of the target DNA (signified by the block grey area). The base sequences of the synthetic oligonucleotides, 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 μ M 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).

non-coding segments of cpDNA may, therefore, be expected to show genetic differences amongst populations that have been genetically isolated for some time.

The overall aim of this work was to develop a simple PCR protocol, which would enable us to investigate genetic variation between plants by using universal primers that are expected to hybridise to the cpDNA of most plants and to use PCR to amplify short lengths of variable cpDNA.

Materials and methods

Polymerase Chain Reaction

The protocol for amplifying plant chloroplast DNA by PCR has three main stages:

1. Extracting DNA from the plants (approximately 50 min).
2. Preparing the extracted DNA for amplification (20 min) and performing PCR using either a thermocycler or water-baths (approximately 90 min).
3. Visualising the amplified DNA fragments by agarose gel electrophoresis (approximately 30 min to prepare and load gels, 30 min to run gel electrophoresis at 100 V or 90 min at 27 V, and 10 min to stain gel).

The sample can be stored in the freezer following Step 1 and following Step 2; the practical can, if desired, be carried out in three distinct sessions. See Box 1 for technical information on this practical.

Extraction and preparation of DNA from plants

DNA was extracted from a leaf disc of approximately 7 mm in diameter. A microcentrifuge tube lid was used to punch out the leaf disc which was added to a microcentrifuge tube containing 200 μ l of 2% SDS in TNE buffer and a pinch of sand. A plastic homogeniser was used to thoroughly grind up the plant material. Cell debris was pelleted by centrifuging the tube for 10 min at 8500 g and the supernatant transferred to a fresh tube. Ethanol (250 μ l, cooled to -20°C) was added to precipitate the DNA from the supernatant. The tube was centrifuged at 8500 g for five minutes to pellet the DNA. The supernatant was discarded and the pellet allowed to dry at room temperature for 10 min. After air drying, the pellet was re-suspended in distilled water (200 μ l) and then stored at -20°C until needed.

Amplifying the DNA using the polymerase chain reaction

10 μ l distilled water, 5 μ l 20 μM CHc primer, 5 μ l 20 μM CHd primer and 5 μ l of extracted DNA sample were added to a PCR tube that contained the ready-to-go pellet and the tube flicked gently to mix and dissolve the pellet. PCR amplification was carried out using a thermocycler or by using a series of three water baths held at 94°C , 55°C and 72°C .

The tubes were initially incubated at 94°C for two minutes and then cycled through the following sequence of three temperatures 30 times:

- 94°C for 30 s
- 55°C for 30 s (temperature was optimised for primers used)
- 72°C for 1 min

Following PCR amplification, the tubes were further incubated at 72°C for 2 min to complete any partially synthesised DNA strands.

PCR samples were stored at -20°C until gel electrophoresis was performed.

Agarose gel electrophoresis

Agarose gel electrophoresis of the PCR amplified DNA was carried out as previously described (see NCBE protocols, www.ncbe.reading.ac.uk/NCBE/PROTOCOLS/menu.html or the Bio-Rad life science education programme, www.bio-rad.co.uk). 2 μ l loading dye was added to 10 μ l of the PCR product and this was loaded on a 1.5% agarose gel prepared using TBE buffer. After electrophoresis in TBE buffer, the gel was stained with Azure A or Fast Blast (see Box 1).

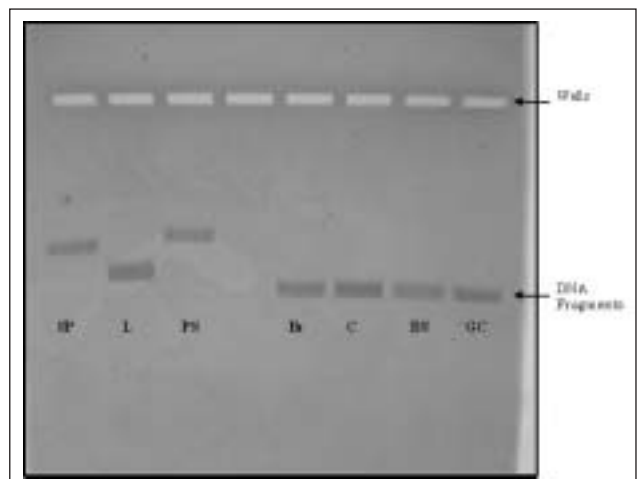
Following this stage, students may analyse the results and draw conclusions regarding the variation between the Brassicas and non-Brassicas. This represents the end of the practical.

Results

In an attempt to devise a simple procedure for the analysis of DNA using PCR within the classroom, we selected abundant chloroplast DNA found in all green plants as a source of DNA. To investigate variation found between plants we selected non-coding regions of chloroplast DNA to amplify by PCR. Non-coding DNA sequences display higher rates of evolutionary change and an increased frequency of mutations, including insertions and deletions of DNA sequences (Soltis *et al*, 1992). For this reason, disparate plant species will often show differences in the length of the non-coding DNA sequence amplified by PCR using primer pairs targeted to the more highly conserved coding sequence.

Here, PCR was carried out to amplify a variable non-coding region of cpDNA found between the highly conserved coding sequences of two tRNA genes, found in the cpDNA of all known green plants. The primers used for PCR were located to the non-variable conserved region of the tRNA gene (see Figure 1). Figure 3 shows the results of PCR amplification carried out on spider plant, iceberg lettuce, purple shamrock, flat leaf parsley, broccoli, cauliflower, Brussels sprout and green cabbage. PCR products from the closely related *Brassica oleracea* varieties (broccoli, cauliflower, Brussels sprout and white cabbage) ran similar distances on the gel, indicating a similar DNA length and suggesting a high level of similarity. In contrast, size variation was observed in the DNA sequences amplified between the different plant genera, as shown by the variation in fragment sizes, which ran different distances on the gel.

Figure 3. Bands of cpDNA stained with Azure A, obtained following PCR of chloroplast DNA extracted from a variety of plant species. (Key: SP – spider plant, L – iceberg lettuce, PS – purple shamrock, Br – broccoli, C – cauliflower, BS – Brussels sprout, GC – green cabbage).



The results shown in Figure 3 were reproduced using either a thermocycler or a series of waterbaths, suggesting this is a robust and reliable protocol to demonstrate variation in plants. The protocol was further tested successfully on a range of plants, which were obtained from a number of sources (see Table 1) and it is thus expected to work on many different plants. We also tested other pairs of primers binding to different areas of the chloroplast DNA and these also worked well. In summary, this DNA extraction and PCR protocol lends itself well to individual projects or investigations, which form part of many post-16 biology courses.

Discussion

PCR amplification of DNA sequences is used in many areas of diagnostic and manipulative molecular biology including DNA profiling (in ecological and forensic contexts), cloning, creating and detecting mutations, detection of genetic diseases and phylogenetics. PCR has revolutionised biology by allowing researchers to generate usable amounts of DNA and simplified many molecular biology protocols. PCR is highly specific and sensitive and has played important roles in many large and small scale projects including the Human Genome Mapping Project. We have devised a simple practical that allows students to learn, discover and experience the general principles of DNA extraction, PCR amplification and separation of DNA fragments by gel electrophoresis. These basic techniques are used in any molecular biology laboratory and the protocols developed in this project can be used to support the Advanced Higher Biology and AS/A2 Level curricula throughout the UK (see Table 2).

While DNA could be isolated from many different sources, multiple copy chloroplast DNA – derived from plentiful and easily obtained green leaf material – provides an excellent and abundant source of DNA to study and to work on within the classroom. In addition, while the technique of

PCR can be used to illustrate many different applications, we have used PCR here to show variation between different plant species, which may be used to enhance teaching of the genetic basis of evolution and speciation. In this protocol, students will extract and use PCR to amplify DNA from a variety of plants that can be obtained from many different sources, but it is important that some are selected from the same genus. In the example described here, we used different varieties from *Brassica oleracea* to show identity and to show variation. The results can be used to highlight genetic similarity and differences within and across genera. They can also help to develop a discussion on the methods by which those genetic differences that result in the formation of individual species may have occurred. These could include mutation (e.g. insertion and deletion), recombination, genetic drift and natural selection. It is important to recognise that this protocol will not detect mutations such as inversions, small insertions or deletions and base pair substitutions.

The protocol can be adapted to develop new primer pairs to characterise further or study different regions of plant chloroplast, mitochondrial or nuclear DNAs. However, it is worth emphasising that cpDNA is highly conserved between species, is maternally inherited and shows limited or no recombination. Therefore the rate of variation in this area of DNA is less than that found in the nuclear genome. This explains why the primers used here work on a wide variety of species.

The National Centre for Biotechnology Education has recently developed this piece of work into a kit (<http://www.ncbe.reading.ac.uk/NCBE/MATERIALS/DNA/ctpcrmodule.html>) which is now available. We believe that with these refinements, made by our colleagues at NCBE, all schools are now able to undertake PCR and show how this modern technique has found applications in a wide variety of areas. Additional materials in the form of photographs related to the techniques used, and Microsoft PowerPoint

Table 2. Curriculum links to UK subject specifications for the protocol, 'PCR of plant chloroplast DNA'

AQA: Assessment and Qualifications Alliance
Edexcel

OCR: Oxford, Cambridge and RSA Examinations

SNAB: Salters Nuffield Advanced Biology

SQA: Scottish Qualifications Authority (AH – Advanced Higher; H – Higher)

WJEC: Welsh Joint Education Committee

Curriculum Authority/Subject	Level	Unit/Module	Curriculum heading/ statement
Scottish Qualifications Authority	AH H	Cell and Molecular Biology Genetics and Adaptation	Applications of DNA technology Variation Selection and speciation
WJEC Advanced GCE Biology	A2	B15 Variety and Control	5.2 Genetic variation is the raw material for evolutionary change
AQA AS/A2 Level Biology A	AS	2: Making use of Biology	11.5 Polymerase Chain Reaction
	A2	5: Inheritance, evolution and ecosystems	14.2 The cause of variation 14.4 The concept of species
AQA AS/A2 Level Biology B	AS	2 Genes and Genetic Engineering	11.4 The Polymerase Chain Reaction
	A2	4: Energy, Control and Continuity	13.10 Causes of variation 13.11 Speciation
Edexcel AS/A2 Level Biology and Biology (Human)		5B Genetics, Evolution and Biodiversity	5B.4 Variation, Gene technology
		5H Genetics, Evolution and Biodiversity	5H.1 Variation, Gene technology
OCR		5.4 2804 Central Concepts (5.4.5) Classification, Selection and Evolution 5.6 2805 Applications of Genetics (5.6.4 Genetic Engineering)	LO (d) Explain the relationship between classification and phylogeny Modification of organisms by genetic engineering
Salters Nuffield	AS	Topic 6: Infection, immunity and forensics	LO 4.6.2 Describe how forensic pathologists determine the identity of a dead person

presentations which might be useful in teaching, will be made available on the Science and Plants for Schools website: www.saps.org.uk

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