The Polymerase Chain Reaction

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THE POLYMERASE CHAIN REACTION

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1. Background information

Deoxyribonucleicacid (DNA) is a complex chemical molecule that is present in all living organisms and carries the genetic information that enables the development and functioning of the whole organism. It is transmitted from parent to offspring, thereby transferring genetic information from one generation to the next. A number of unique features of the molecule allow it to perform this function. DNA usually exists as two parallel strands of nucleic acids. Each strand is a long polymer of four bases – Adenine (A), Thymine (T), Cytosine (C) and Guanine (G). The order of the individual bases within each strand is very variable. However, because of the structure of DNA the bases in one strand pair with those in the opposite strand. Adenine pairing exclusively with Thymine, and Cytosine pairing exclusively with Guanine. The variation in the order of the bases enables vast amounts of information to be stored, and exact base pairing ensures that the information can be precisely copied. Each strand of DNA contains all the information needed to build its complementary (opposite) strand.

2. Description of PCR

The Polymerase Chain Reaction (PCR) is based upon DNA's characteristics of base pairing and precise copying of complementary DNA strands, PCR allows the researcher to make numerous copies of interesting regions of DNA molecules.

The PCR uses the ability of a small piece of DNA (a primer) to find and bind to a target sequence, its complementary sequence, on another DNA strand. Then an enzyme called a polymerase adds individual nucleotides to the end of the primer to make a new piece of DNA that is complementary to the DNA target sequence. By repeating this process several times, the target DNA sequence is amplified exponentially.

In the basic form of PCR the researcher has to know the sequence of the bases on either side of the target DNA before this DNA can be amplified. The researcher then makes primers that are complementary to these sequences. Amplification can be achieved in a small volume of liquid (50 μl), to which the researcher adds the DNA to be copied, the individual nucleoticle bases (A, T, G, C), the primer, the polymerase enzyme and a buffer to maintain optimal conditions for the reaction. The mixture is heated, which separates the two strands of the target DNA, normally held together by bonds between complementary bases. The mixture is cooled and the primers find their complementary sequences on the long strands of DNA and bind to them. The polymerase enzyme locates where the primers have bound to the DNA and joins individual bases to the end of the primer to form a new strand of DNA. The polymerase uses the target DNA as a template, each base that is added to the new DNA will be complementary to its opposite number in the target sequence. The sequence of the new DNA is exactly complementary to the sequence of the target DNA, and the two strands are bound together.

After a while, when the researcher can be reasonably sure that the new DNA strand has been extended as far as the primer sequence at the other end of the target DNA, the mixture is heated again. The newly synthesised DNA and the original DNA separate into single strands, but where originally there were just two copies of the target, now there are four. Again, as the mixture cools, the primer binds to the target sequence, and the polymerase builds a new strand of DNA on the target, producing eight copies of the target. Each new cycle of heating, cooling and polymerisation doubles the number of copies of the target DNA.

Furthermore, the ends of all of the copies are the same, since their ends are defined by the oligonucleotide primers. This means that after PCR, the researcher can purify the amplified sequence by separating the DNA in an agarose gel with the aid of an electric current. This sorts DNA by size; since all the copies of the target DNA are the same length, they will lie in a neat band on the gel.

Under ideal conditions, the number of copies doubles with each round of denaturing, annealing, and polymerisation. After 30 cycles there will be more than a billion copies.

3. Important stages in the development of PCR

Initially, although the technique proved valuable, it was laborious. The temperature required to separate the complementary strands of target DNA also destroyed the existing polymerase enzymes. Fresh enzyme had to be added to the reaction after every amplification cycle, necessitating the continual presence of the operator. The inventor of the PCR identified and isolated a thermally stable polymerase in *Thermophilus aquaticus*, a bacteria that lives in hot water springs. This polymerase enzyme (nicknamed *Taq* polymerase) can withstand the temperature required to separate the DNA strands, thus, once the reaction mixture has been prepared, it can be left unattended and will amplify DNA through >40 cycles of heating and cooling without the need to add further enzyme.

This development facilitates the automation of PCR. Temperature cycling machines that heat or cool the reaction mixtures to the desired temperature for each stage of the amplification process, and maintain the required temperature while each stage is completed, are now widely available.

The ability of *Taq* polymerase to withstand high temperatures also enabled scientists to carry out the annealing step of the reaction, during which the primer binds to its target sequence at a higher temperature. The higher the temperature at this stage, the more precise the match between the primer and the sample DNA must be before they can bind together. This improved the sensitivity of PCR by ensuring that the primer had found its intended target, not another sequence that was a close but not a perfect match.

4. The advantages of PCR

a) *High sensitivity*. PCR can amplify many copies of the desired sequence even when there is only one copy of this sequence in the target DNA.

b) *Specificity*. Under optimal conditions correctly designed primers will only amplify the desired target sequence.

c) *Exact copying*. The amplified DNA will have the same sequence of genetic information as the target sequence. Errors during copying are rare, but can be induced by varying the reaction mixture parameters if required.

d) *Speed*. High copy numbers can be reached within a few hours.

e) *Simplicity*. Once reaction conditions and primer sequences have been optimised, preparation of reaction mixtures and purification of amplified sample is very simple.

Other techniques of genetic analysis can take 7–10 days to perform; they are more complex experimentally and require much larger quantities of target DNA.

5. The problem of contamination

Whilst being its major advantage, the ability of PCR to amplify very small quantities of DNA is also a potential disadvantage. PCR will amplify any DNA that contains a suitable target sequence. Therefore, if the sample DNA, or any of the other ingredients, are contaminated with DNA from another source, the reaction can amplify sequences from the contaminating DNA and produce false positive results. This problem can be overcome by ensuring absolute cleanliness in laboratory practices and by using equipment and work areas dedicated solely for PCR. Ideally such areas should be segregated from other laboratory areas. To verify that experiments are not contaminated, they must be designed to include adequate controls which can confirm the DNA that is amplified is indeed the sample.

6. The availability of equipment

It is now relatively simple to initiate research utilising PCR. The reagents and machinery required are widely available and there are a wealth of publications detailing the parameters for successful PCR. A survey of the suppliers catalogues in one department of SCRI revealed 10 manufacturers marketing 14 models of PCR machine and 6 companies selling *Taq* polymerase. It is also possible to purchase off the shelf kits that contain all of the "ingredients" and "recipes" necessary to start PCR research.

7. The uses of PCR

The ability of PCR to take small quantities of DNA material, copy them precisely until large amounts are produced, has had an enormous impact in all sectors of biology. Listing all of the uses of PCR would use too much space; however, major achievements in some areas are outlined below.

Diagnosis of genetic disease

PCR has facilitated important advances in the diagnosis of genetic diseases where early diagnosis is beneficial. The speed and reliability of PCR has enabled the detection of particular genes in individual cells from developing human embryos. It is possible to identify embryos carrying genes for sickle cell anaemia, the genes for cystic fibrosis, and defects in the gene for the blood clotting factor IX which can cause haemophilia. The speed of the system allows the possibility of safer and less traumatic electric abortion and the selection of embryos that do not carry genetic defects for *in vitro* fertilisation

AIDS research

The virus that causes AIDS can be present at very low levels in an individual's DNA. PCR allows the virus to be amplified thereby facilitating its detection. This is especially useful in the diagnosis of HIV in babies. It may allow doctors to see if certain drugs can eradicate HIV infection. PCR also allows the detection of a form of pneumonia (PCP) that only infects

people with a weak immune response and is often the first sign of AIDS. In previous tests for PCP large samples of lung tissue had to be removed for analysis by a painful procedure using a bronchoscope. The PCR based test needs only a small amount of saliva and mucus from the back of the throat, which can be obtained painlessly in minutes.

Forensic analysis

PCR has revolutionised forensic medicine by allowing a person's unique genetic fingerprint to be obtained from the DNA in minute amounts of hair, blood, skin and semen. It has provided vital evidence determining conviction or acquittal in rape and murder cases.

Archaeology

DNA can be extracted from archaeological remains such as Egyptian mummies, mammoths frozen in ice, steel knives and bones in burial mounds. Analysis of the DNA by PCR has produced data on the social structure of prehistoric colonies, their hunting practices and interactions with other tribes. Analysis of specific gene sequences from archaeological remains is revealing how genes have changed (evolved) over time periods of thousands of years.

8. Extending the usefulness of PCR

The standard form of PCR is a very powerful technique for amplifying certain target sequences of DNA. It is limited, however, to amplifying stretches of DNA that are delineated by known stretches of DNA to which primers with complementary sequences can be made. Researchers have modified the standard form of PCR to extend its range of applications even further.

a) Inverse PCR

One drawback of standard PCR is that the polymerase builds a new DNA strand from one primer across the target sequence towards the other primer. Thus, the ends of the target are defined by the primer sequences but often regions of interest lie on the side of the target. Such regions might be areas of DNA that promote the expression of the target gene or areas which determine how the gene product is modified once it is expressed (processors). Researchers in the USA have devised a method whereby the target DNA is cut into small fragments without cutting within or near to the target sequence, and the ends of the DNA are induced to come together to form circular DNA molecules. If a PCR reaction is conducted on these molecules, the primers will match the end of the target sequence but will be facing in the other direction, thus the polymerase works in the other direction and the DNA of the regions flanking the gene of interest are amplified and can be studied to determine how they affect the expression of the gene.

b) Anchored PCR

To conduct standard PCR on a target sequence, the sequences at both ends of the target must be known to allow complementary primers to be synthesised. Often scientists only know the sequence of the DNA at one end of their target. However, the technique of anchored PCR allows them to attach a short piece of DNA of known sequence (an "anchor") to the unknown

end of the DNA they are interested in; then primers with complementary sequences to both ends of the target sequence can be synthesised and the target successfully amplified by PCR.

c) Random Amplified Polymorphic DNA PCR (RAPID–PCR)

In extreme cases scientists may wish to study the differences between individuals but has no idea which genes will be of interest, or do not know the DNA sequence at either end of the target gene. In this instance PCR can still prove extremely useful. Primers can be synthesised where the base sequence is chosen at random. These random primers will bind to sample DNA, where complementary sequences are present and the PCR reaction will amplify pieces of DNA that have been chosen at random from within the sample DNA. Between 50 and 98% of random primers generated in this way produce useful differences in the amplified DNA products, depending on the species being studies and the nature of the investigation.

d) Reverse Transcriptase – PCR (RT–PCR)

The versions of PCR described so far can be used to study the genetic constitution of an individual by describing the genes that are or are not present in the individual. Although genes are present in an individual they are not always expressed. An important area of biological research is the investigation of the differences in gene expression during the life cycle of an organism, its development to maturity, its senescence or during disease. Gene expression involves two distinct phases. During the translation phase the double helix of DNA is uncoiled, becomes single stranded and each strand is converted into another form of nucleic acid, messenger ribonucleic acid (mRNA). The structure of mRNA is similar to that of DNA. mRNA is a string of nucleic acids (bases) and, during the formation of mRNA, each base binds to its complementary base in the DNA and the bases are joined together by an enzyme called transcriptase. The function of transcriptase is similar to DNA polymerases although the former produce RNA from a DNA template. In the second stage of gene expression the mRNA is converted into proteins (enzymes) that affect the biochemistry, physiology and appearance of the individual.

mRNA can be extracted from an organism. This extract represents the genes that were being expressed at the moment the extract was being made. However, mRNA is relatively unstable and occurs at low concentrations within the organism making it difficult to study. Researchers have found that by incubating the mRNA extracts with an enzyme called reverse transcriptase, mRNA can be converted back to DNA. This enzyme has the opposite effect of transcriptase and, because of the precise nature of complementary base pairing, its action on mRNA samples produces the DNA that was being expressed. Scientists can take this DNA and amplify it by one of the forms of PCR to produce large quantities of the less labile DNA. This DNA can be studied to determine how numerous abiotic and biotic factors influence gene expression. The technique of RT–PCR is likely to be invaluable in determining the control of developmental processes and the events that occur during disease.

9. The uses of PCR at SCRI

PCR is used in seven of the nine science departments at SCRI. Part of the work of one of the other departments is the production of PCR primers for use within SCRI.

The use of PCR at SCRI has expanded rapidly in the last three years and this trend will continue. There are currently 20 PCR machines at SCRI and the institute is planning to purchase at least three more machines in the immediate future.

There are two broad areas of research where PCR is an essential part of the work. Firstly, RAPID–PCR is widely used to study biodiversity, population structure and evolutionary trends. Secondly, PCR is used in all studies of gene structure, regulation and expression. PCR is also used as a general, every day, laboratory process to facilitate other research, for example to multiply copies of a DNA sequence for use in other procedures.

The uses of PCR can be reviewed on a departmental basis.

Cellular and Environmental Physiology

Studies of gene expression using PCR and RT–PCR aim to characterise gene regulation in important areas of plant physiology; the induction of tuber formation in potatoes; carbohydrate metabolism in potato tubers and seeds of other plant species; cold indictable genes involved in the dormancy of tree seeds; the metabolic pathways in blackcurrant ripening. Future work will use RT–PCR and PCR amplified gene libraries to investigate the tissue specific expression and regulation of plant genes.

PCR is used for routine laboratory tasks in gene isolation (cloning); putting primer linkers into isolated gene constructs; routine checking of insert sizes in gene libraries and for amplifying larger quantities of DNA.

Cell and Molecular Genetics

PCR is used in **all** of the SOAFD commissioned work in this department, and the majority of the externally funded work. This department has 11 PCR machines and is planning to buy more.

RAPID–PCR is extensively used in

i) gene mapping and linkage studies that aim to locate molecular markers closely associated with useful genes. Markers linked to genes of interest can increase the efficiency of selection in plant breeding programmes and can facilitate gene isolation;

ii) biodiversity and evolutionary investigations. This work assesses the level of genetic diversity within germplasm resources of wild and cultivated forms of important crop species (e.g. potato), and investigates the evolutionary trends within plant taxa.

Much of the work of this department involves the introduction of genes into plants either to confer additional agronomically useful characteristics to that genotype, or to study the effect of the gene on the biochemistry, physiology or phenotype of the plant. PCR is used as a quick method of identifying transgenic plants into which the genes have been successfully introduced. This forms an important part of studies into low temperature induced changes in carbohydrate metabolism in potatoes.

PCR, inverse PCR and RT–PCR are being used in studies of the molecular regulation of gene

expression including work on pre-messenger RNA processing.

Chemistry

The Chemistry department synthesises primers for much of the PCR work at SCRI. This ensures the rapid availability of primers with unique sequences for use by SCRI scientists.

Crop Genetics

Undertakes a large amount of genome mapping and linkage studies of wheat barley and Faba bean in collaboration with the Cell and Molecular Genetics Department.

The department also uses RAPID-PCR as environmentally benign markers in risk assessment projects to assess the likelihood of gene escape from genetically modified organisms, and to characterise protoplast fusion products. Also, using RAPID-PCR to study the introgression of wild species DNA into cultivated forms of potato by the novel mechanism of inter-species somatic recombination.

Mycology and Bacteriology Department

This department uses RAPID-PCR to study the genetics of population structure; sexual recombination within pathogen populations; and will use this technique in the near future for genomic mapping of pathogen species. This work aims to describe the evolution and changes in host specificity in plant pathogens thereby providing knowledge to underpin the development of sustainable disease resistance in plants.

The department has funding from the Horticultural Development Council to develop PCR technology to enable the detection of plant pathogens in vegetatively propagated crops such as hardy ornamentals, strawberries, raspberries, and some vegetable crops. These detection systems would improve the health status of such crops and increase the efficiency of phytosanitary schemes.

As part of one project, scientists are using PCR and RT-PCR to isolate a proteinase inhibition gene from raspberries which may have an important role in resistance to the grey mould pathogen (*Botrytis cinerea*).

Soft Fruit Genetics

RAPID-PCR is being used to "fingerprint" plant germplasm as an aid to varietal identification and checking trueness to type of clonally propagated material.

Various forms of PCR are being used to identify and isolate genes that are likely to be of significant use in the improvement of soft fruit crop species. Target genes include those conferring physical attributes (e.g. thornlessness) and disease resistance (e.g. raspberry root rot *Phytophthora fragerii* var. *rubi*).

PCR is also used to confirm that genes have been successfully introduced into transformed plant material. The main thrust of this work is to introduce the cow pea trypsin inhibitor gene into soft fruit crops, thereby increasing resistance to insect pests.

Virology

PCR is used in approximately two-thirds of the SOAFD commissioned work within the Virology Department. It is used as a routine technique in a number of project areas:

i) a sensitive detection method to confirm the presence of a virus in a plant or in a virus vector (aphids, fungi, nematodes). PCR is used to detect DNA viruses, RT-PCR to detect RNA viruses.

ii) a diagnostic tool to distinguish different viruses or virus strains in host plants or their vectors. This is an important part of host-specificity and virus epidemiology studies.

iii) to detect the presence of artificially introduced genes in transgenic plants. There is a major project investigating the production of transgenic plants containing genes that code for virus coat proteins and the effectiveness of this strategy as a method of generating virus resistant plants.

iv) PCR is used in the cloning of antibody genes and the manipulation of these genes to produce recombinant antibodies (novel serological reagents). These are likely to be important in the immunodiagnostics and host resistance to plant viruses.

v) PCR, in conjunction with DNA sequencing, has been used on a small scale for phytogenic studies of the relationships between virus strains.

PCR is used as a routine laboratory tool in a number of projects for increasing yields of DNA, and checking of gene libraries.

Zoology

RAPID-PCR is used to study the evolutionary trends and relationships within and between plant pathogenic nematode populations in the UK. For example, all potato cyst nematode populations in the UK were introduced from South America. RAPID-PCR is providing insights into the source and spread of nematode types and the reduction in heterogeneity of the nematode population during spread within the UK. This has revealed the prevalence of resistance breaking forms throughout the UK.

The Zoology department plans to use RT-PCR for gene isolation studies. For example, collagen coats parts of the nematode feeding apparatus that are important for plant virus retention and transmission. Isolation of these genes will give a greater understanding of the nature and specificity of virus transmission by plant parasitic nematodes.

To summarise, the various forms of PCR are a vital constituent of numerous aspects of the research at SCRI. PCR is the basis of many of the research projects and is an essential adjunct to numerous others.

10. The impact of licensing and royalty charges

The scientific community regard PCR as a basic scientific tool, similar to a centrifuge or a pipette. Any restriction on the use of PCR would have an immediate, detrimental effect on

fundamental and applied research programmes at SCRI. The current position regarding the use of PCR for scientific research is unclear and needs to be clarified immediately. Licensing individual users would be a time consuming and costly operation. The crown should enter into negotiations on behalf of all government departments and attempt to obtain a single licence.

A recent article in the Financial Times suggested that Roche have offered freedom to use PCR in human *in vitro* diagnostic testing. The position regarding the use of PCR in plant pathogen diagnostic testing and the fingerprinting of cultivars for trueness–to–type must also be clarified. If scientists at SCRI were to develop PCR based systems of pathogen detection or cultivar fingerprinting, with whom would the intellectual property rights lie and would a proportion of any profits made as a result of this research have to be given to Roche?

The thousands of scientific publications on PCR and the widespread availability of PCR machines, *Taq* polymerase and reagents required for PCR may make it difficult for Roche to control the market as tightly as they would like.

The concerted action of the British Government is needed to clarify the legal position regarding licensing and intellectual property rights and ensure that negotiations are conducted from a position of strength.

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