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The PCR Revolution

Rosalind A. Eeles, William Warren and Alasdair Stamps

"Behold, how great a matter a little fire kindleth."
James, III:5

INTRODUCTION

THE POLYMERASE chain reaction (PCR) is an *in vitro* method which uses enzymatic synthesis to amplify, exponentially, specific DNA sequences. Its specificity is equivalent to finding the needle in the proverbial haystack and the amplification so powerful that it is equivalent to taking one person and cloning them to repopulate the UK. With PCR, molecular biologists now have a very powerful tool to amplify specific regions of DNA within the DNA extracted from a single cell or cells, and examine the sequence of that region. This technique has multiple applications, some of which will be outlined in this review.

The importance of this technique is reflected by the exponential increase in the number of publications relating to PCR, from three in 1986 to a staggering 1700 in 1990. These include several reviews [1–4] and excellent texts [5, 6].

The revolution was made possible firstly by the introduction of a thermostable enzyme, Taq polymerase which remains active at the high temperatures used in part of the PCR cycle and secondly by microchip technology which enabled the development of automatic cycling machines. PCR overcomes the problems associated with minute sampling; Southern blotting requires 10 μg of DNA whereas PCR only requires one copy of the relevant gene to be present. PCR is an extremely rapid technique, taking only about 2 h to complete, so diagnosis of genetic abnormalities can be made within one day as opposed to 3 or 4 days when Southern blotting techniques are used.

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THE POLYMERASE CHAIN REACTION

The standard reaction uses two oligonucleotide primers which are complementary to and hybridise to opposite DNA strands flanking the region of interest in the target DNA. The primers are generally around 20 nucleotides in length, sufficiently long to be unique within the genome.

The reaction which is shown diagramatically in Fig. 1, consists of the following steps:

1. Template denaturation —	at 90–94°C for 0.5–2 min: this separates the two DNA strands.
2. Primer annealing —	at 50–60°C for 0.5–1 min; the primers anneal to the template (the annealing temperature depends on the nucleotide composition of the primers).
3. Extension —	at 72°C; new DNA strands are synthesised from the primers, complementary to the single stranded template DNA to which the primer has hybridised.

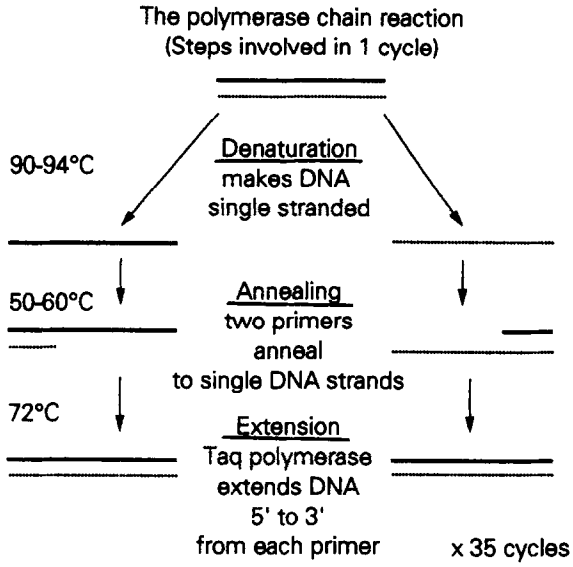

Fig. 1. The polymerase chain reaction. Steps involved in one cycle. —— single DNA strand, - - - - - complementary DNA strand.

The extension step is usually programmed to last 60–90 sec, but extension occurs very quickly (approximately 24 nucleotides/sec) and this step can be omitted if the PCR product is ≤ 150 base pairs in size.

These steps are then repeated, i.e. the newly synthesised DNA strands are also available as templates for a further round of DNA synthesis in the next cycle of the reaction. The DNA is therefore amplified exponentially, theoretically 2^n times where n is the number of cycles, although in practice the efficiency is not 100%. Typically 30–40 cycles are performed and although it is easier to amplify about 500 base pairs, up to 10 kilobases can be amplified. However, the larger the amplification product, the larger the number of shorter nonspecific sequences which may also be amplified.

The polymerase chain reaction was originally devised by Mullis and was refined by Saiki *et al.* in 1985 [7]. It originally used *Escherichia coli* DNA polymerase which was not heat stable, so fresh enzyme had to be added before each cycle. Water baths were used to heat and cool the reaction mix, and the various steps timed using stop watches. This procedure was time consuming and arduous to perform. The PCR revolution followed the development of computerised thermal cyclers which automatically heat and cool the samples, and the introduction of a thermostable Taq polymerase [8], isolated from algae (*Thermus aquaticus*) living in the hot springs of Yellowstone National Park.

The 'standard' PCR

No set of conditions works for every pair of primers and template, but the following is adequate for most situations. The final reaction volume is usually 25–100 μl and contains:

Buffer	50 mmol/l KCl 10 mmol/l Tris HCl (pH 8.4 at 37°C) (some experimenters also add Tween 20 at 0.05%)
DNA	100 ng (smaller quantities may be used)
$MgCl_2$: final concentration:	0.5–3.0 mmol/l
(the optimal Mg concentration varies for different pairs of primers)	
dNTPs	at 200 μmol/l each: (dATP, dCTP, dGTP, dTTP)
Primer 1	0.25 μmol/l
Primer 2	0.25 μmol/l
Taq polymerase	1–2 units
Deionised water	to make up to final volume

A drop of mineral oil is added to the top of the reaction mix to seal it and prevent evaporation.

This protocol can be modified to amplify DNA from paraffin sections [9–11], and even single cells [12]. PCR protocols have also been developed to amplify DNA from whole blood, without first extracting DNA [13].

A plateau effect occurs as the efficiency of the reaction decreases with increasing numbers of cycles. However, the reaction can be made quantitative ('quantitative PCR') by keeping the number of cycles within the linear range of amplification and including in the reaction a control DNA fragment amplified by the same primers, giving a PCR product of similar, but distinguishable length compared with that of the target sequence. This can be used to quantify RNA expression [14].

Choosing primers

These are usually around 20 nucleotides long, and each is complementary to a sequence on one of the two DNA strands either side of the target sequence. DNA is synthesised 5′ to 3′ and when ordering primers, the sequences are generally written in this order. An example is given in Fig. 2.

The primers should ideally have about the same proportion of purines and pyrimidines to reduce self-complementarity and stretches of the same base should be avoided. Computer programmes can be used to ensure that sequences do not have a significant secondary structure. The primers should not contain complementary regions to minimise the formation of 'primer dimers' (where the primers hybridise to each other rather than the denatured DNA to be amplified).

Specific DNA sequences, such as restriction enzyme sites, can be attached to the 5′ end of the primer to facilitate cloning into vectors.

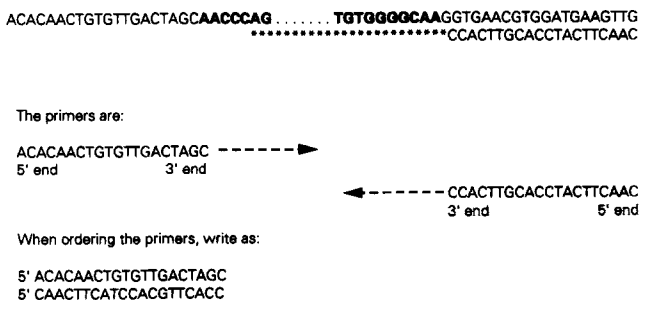

Fig. 2. DNA sequence to be amplified is in bold type with the DNA sequence either side of it in ordinary type. *** is the complementary DNA strand in the DNA to be amplified.**

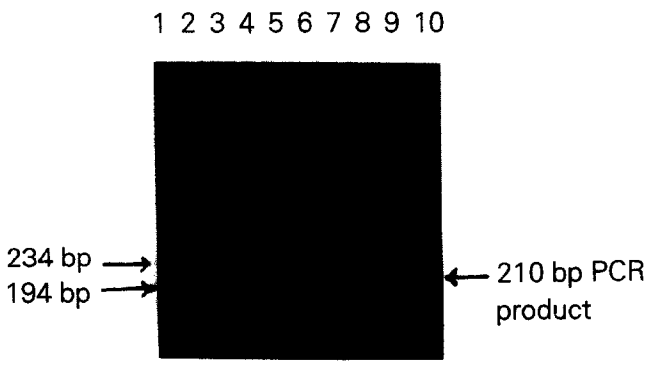

Fig. 3. Ethidium bromide—stained 2% agarose gel. PCR products, each of 210 base pairs in lanes 2–9. Lane 10 is a negative control (no DNA to demonstrate that there is no PCR contamination), Lane 1: ϕ × 174 bacterial digested DNA, digested into pieces of known size to act as a size marker.

The precision of the reaction

Non-specific primer binding can lead to the formation of nonspecific amplified portions of DNA. This can be minimised as discussed in the 'problems' section. Alternatively, two polymerase chain reactions can be carried out, the second of which uses nested primers which lie 3′ to the original primers. Taq polymerase lacks proof reading ability, introducing errors at a rate of about 3 per 1000 nucleotides incorporated.

Problems

The greatest problem with PCR is contamination of newly set-up reactions by products from previous reactions. An analogy for the potential scale of the problem is as follows: if the products of a 100 μl PCR were added to an Olympic-sized swimming pool, then a 0.1 ml aliquot from this pool would contain 40 amplifiable molecules [15]. Contamination can be minimised by aliquoting solutions and using separate pipettes and solutions for pre- and post-PCR experiments. Some experimenters use positive displacement pipettes or plugged pipette tips which are now available to eliminate contamination due to the production of aerosols. Many laboratories also have designated areas for handling PCR products. Negative controls should always be included in each PCR experiment to monitor potential PCR contamination, and of course, the control reactions should be set up last.

Nonspecific amplification can be reduced by increasing the annealing temperature, reducing the amount of template DNA, lowering the magnesium concentration, using 10% dimethylsulphoxide (DMSO) or 1.25–5% formamide [16].

Analysis of PCR products

The PCR products are usually visualised by electrophoresis in agarose and staining the DNA with ethidium bromide. The products can then be viewed under ultraviolet light (Fig. 3). The products can also be immobilised on filters and hybridised with radioactive probes. Alternatively, a radioactive product can be generated by including labelled nucleotides in the PCR, or by labelling one of the primers. In addition, ligands can be incorporated into the primers enabling colorimetric detection [17].

PCR products can be sequenced after cloning, but if this method is used, only one amplified sequence is examined, and misincorporation of bases by the Taq polymerase may result in errors. This problem is overcome by direct sequencing of PCR products; individual mismatches then become an insignificant proportion of the total number of sequenced molecules. Direct sequencing from PCR products is also more rapid. Methods include either sequencing from single stranded DNA created by asymmetric PCR [18], or the isolation of single-stranded DNA from the double stranded PCR products, by using biotin-labelled primers and magnetic separation technology [19].

APPLICATIONS OF THE POLYMERASE CHAIN REACTION

Infectious diseases

Viral DNA can be detected by PCR for diagnosis, such as in cases of the acquired immunodeficiency syndrome [20] where the patient is still antibody negative, or in babies born to HIV positive mothers who will have acquired antibodies across the placenta, but may not necessarily be infected with the virus [21]. The detection of viral DNA is also applicable to paraffin sections [22].

Forensic science/anthropology

The advantage of PCR in this situation is its ability to amplify minute amounts of DNA, such as may be obtained from a single human hair or a blood spot [23, 24], to a level that is sufficient for analysis. Within the genome of eukaryotes, between the sequences that are translated into protein (the exons), lie noncoding regions (the introns). Within the introns are runs of repeated base sequences. Some are end to end repeats of sequences of about 30 bases; the numbers of times these sets of bases are repeated vary between different individuals: they are therefore called VNTRs (variable number of tandem repeats) or minisatellites. Up to six minisatellites can be co-amplified in a single PCR reaction [25]. The pattern of repeat sequences is called the genetic fingerprint of the person tested, and the combination of repeats is virtually unique for each person, making this technique invaluable in forensic science [26].

The stability of DNA, even over many years, has enabled it to be analysed from ancient specimens which can aid anthropological study [27].

Haematology

Sickle cell anaemia was the first disease to which PCR was applied for prenatal diagnosis [7]. The genetic defects in haemophilia have been detected using 'chemical mismatch analysis' of PCR products, and show whether cases arise as a result of new or inherited mutations [28]. PCR has been used in the diagnosis and follow-up of many haematological malignancies, as outlined below.

Cancer diagnosis, treatment and research/clinical genetics

PCR can be used for diagnosis or the detection of minimal residual disease. Certain chromosomal translocations commmonly occur in follicular non-Hodgkin lymphomas (t(14;18)) and chronic myeloid leukaemia (Philadelphia chromosome positive: t(9;22)). In follicular non-Hodgkin lymphomas, the translocation involves the immunoglobulin heavy chain region on chromosome 14 and the bcl-2 region (a putative oncogene) on chromosome 18. The primers used hybridise to the regions flanking the translocation and will therefore only amplify the intervening DNA when the translocation is present. This can be used both for diagnosis and the detection of minimal residual disease, even in the presence of a histologically negative bone marrow [29]. Dilution experiments show that PCR assays containing 10 μg of DNA can nonetheless detect a single lymphoma cell amongst 1×10^6 normal cells. This is more than

a 10^4-fold improvement in sensitivity over Southern blotting methods. In the case of chronic myeloid leukaemia, the breakpoint on chromosome 9 can occur over a large area, and the potentially large fragments cannot be amplified. This problem can be overcome by basing the amplification on mRNA, rather than genomic DNA. This is performed on cDNA synthesised from mRNA by reverse transcriptase [30].

The presence of viral DNA in precancerous lesions has also been detected using PCR [31].

Detection of point mutations associated with a particular disease process. Point mutations are one mechanism for both oncogene activation and tumour suppressor gene inactivation. Ras activation, for example, is due to point mutations within certain codons of the gene, notably codons 12, 13 and 61. Following amplification of the relevant regions of the *ras* gene, these mutations may be detected by a variety of methods [32–35]. These analyses have now been performed on many tumour types to ascertain the type, and timing, of mutations involved in carcinogenesis.

Prenatal diagnosis

This can be performed on samples taken at amniocentesis or chorionic villous sampling [36–39]. The fact that PCR requires only minute samples is particularly advantageous in this situation.

Genetic linkage studies

Amplification of minisatellites, as used for DNA fingerprinting in forensic science, can also be used in linkage studies to identify disease loci. Microsatellites, which are highly polymorphic stretches of variable numbers of dinucleotide repeats (such as CACACA), can also be amplified by PCR and used as markers for linkage. Their high degree of polymorphism renders them particularly informative in linkage analysis. These markers are being used to search for genes which may predispose to some forms of cancer.

Gene cloning and sequencing

DNA clones, either phage plaques or bacterial colonies, can be directly characterised by PCR, allowing the rapid screening of DNA libraries without the need for plating [40]. Inserts can be amplified directly from plasmids without restriction enzyme digestion and purification [41]. Primers can be constructed with restriction sites on their 5′ ends so that these sites are present on the amplified product and it can easily be cloned into vectors [42].

Probing gene families

Related genes, both between species and within one species (e.g. the tyrosine kinase family of genes), have shared (conserved) sequences. To discover new, but related genes, one can design degenerate primers which correspond with all possible nucleotide sequences which code for the protein in question, and one can introduce further degeneracy in order to encompass related protein sequences. Using a lower annealing temperature, with conventional primers is another way of amplifying 'related' sequences. Conventionally, 'degenerate' or 'low-stringency' hybridisation methods are used with a labelled oligonucleotide on a standard genomic library [43].

FURTHER DEVELOPMENTS

The PCR revolution has occurred because of the application of computer technology and a thermostable DNA polymerase to the cyclical amplification of DNA. Its power of amplification from minute samples has opened up enormous possibilitiies for rapid diagnosis, forensic science and research. Further refinements of existing diagnostic methods to incorporate the benefits of PCR will lead to even wider use of this technique in the next few years.

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The p53 Gene in Human Cancer

M. R. Stratton

INTRODUCTION

CERTAIN DNA viruses can induce tumours in experimental models. The transforming activity of the small DNA virus SV40 is attributable to a portion of the viral genome that encodes a protein termed the large T antigen. p53 was initially detected as a host cell protein that associates with the large T antigen in cells infected by SV40 [1, 2]. It was independently discovered as an antigen overexpressed in chemically transformed mouse cells [3].

STRUCTURAL ALTERATIONS IN THE p53 GENE AS SOMATIC EVENTS IN HUMAN CANCER

The first indication that mutation of the p53 gene might be a common step in the development of human cancer emerged from investigations into the role and location of tumour suppressor genes (recessive oncogenes). Comparison of germline and tumour DNAs using polymorphic probes revealed that loss of one or other parental allele (loss of heterozygosity) on the short arm of chromosome 17 occurs at high frequency in many types of neoplasm. This type of result is usually interpreted as indicating the presence of a tumour suppressor gene in the vicinity. The p53 gene had previously been localised to this region and fine mapping of deletions in colon carcinoma indicated that the common deleted area includes the p53 locus [4]. Prompted by this clue, Vogelstein's group sequenced the p53 gene in two colon carcinomas and subsequently in other tumours showing loss of heterozygosity on chromosome 17p. In most cases single base substitutions were detected in the remaining p53 allele [4, 5].

A substantial body of data contributed by several groups now indicates that the p53 gene is the most commonly mutated gene known in human cancer. Most of the mutations are missense single base substitutions resulting in replacement of one amino-acid by another in the p53 protein. A minority result in abnormalities of mRNA processing, frame shifts or premature termin-

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