Polymerase chain reaction

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The polymerase chain reaction (PCR) is a technique for rapid amplification of a selected region of DNA, generally where the sequence at both 5′ and 3′ ends is known. In recent years it has been used for an extensive and ever-expanding range of biological applications, which include amplification of RNA sequences that have first been reverse-transcribed into DNA (RT-PCR).

PRINCIPLE OF PCR

PCR depends on generation of a deoxyoligonucleotide complementary to the known 3′ terminus of the portion of DNA to be amplified. This is then annealed with the appropriate strand of heat-denatured DNA and used as a primer for synthesis by a DNA polymerase of a full-length complementary strand (Fig. 1). The two strands are then separated by heat denaturation and the process repeated. However, included within the incubation mixture is a second primer capable of hybridizing to the 3′ end of the complementary strand of DNA. Thus, during cycles of denaturation, annealing and DNA extension, both the forward and reverse strands of the initial template DNA can act as templates for synthesis of new complementary strands. Since at the end of a cycle, each of the original complementary strands has generated its own complement, each

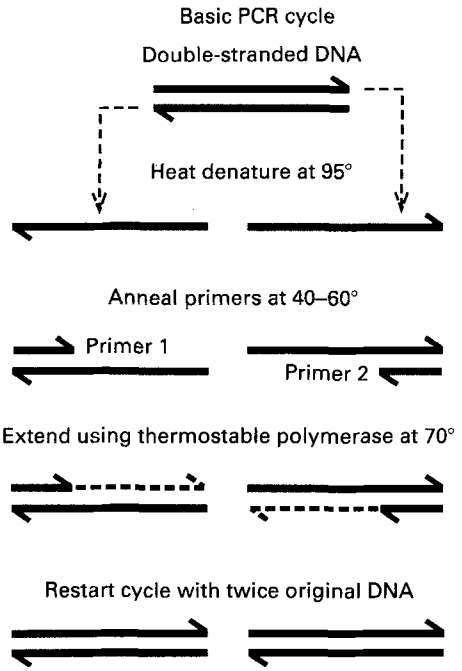

Fig. 1. Basic principle of DNA amplification during one polymerase-chain-reaction (PCR) cycle.

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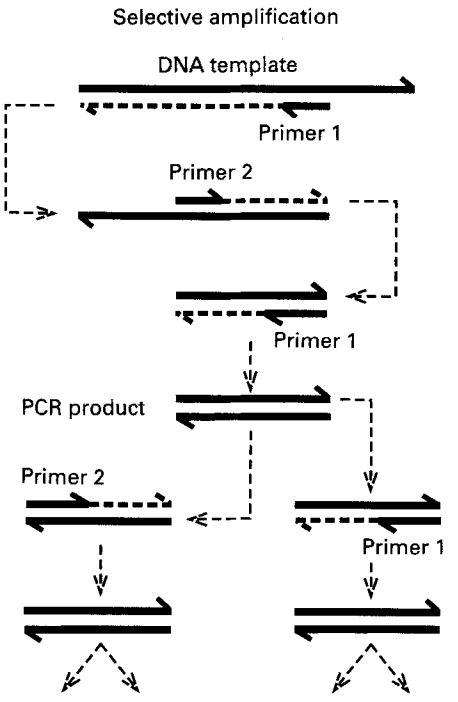

Fig. 2. Selective amplification of a section of DNA lying between two polymerase-chain-reaction (PCR) primers.

cycle doubles the quantity of amplified DNA present and the amplification proceeds logarithmically. Thus, theoretically thirty cycles of amplification would generate nearly 10^9 times the original quantity of DNA.

The section of DNA amplified need only be a relatively small fraction of the initial template DNA. Thus, provided the DNA is accessible both to the primers and to the polymerase, small sections within a strand of chromosomal DNA can be selectively amplified with primers which anneal to the 3′ termini of the complementary strands of the section of interest (Fig. 2). Furthermore, the starting material may be RNA rather than DNA if the initial cycle utilizes a reverse transcriptase rather than DNA polymerase to extend the primer.

APPLICATIONS

Table 1 lists a selection of applications involving PCR which have relevance to nutritional studies. The technique may be used for preparation of labelled-DNA probes without having first to isolate the relevant section of the DNA from its plasmid or genomic DNA (Hirst *et al.* 1992). This can facilitate the production of probes for Northern- and Southern-blot analysis (see Kelly, 1996).

Families of genes having similar sequences can be investigated with generic primers often produced with a degree of in-built degeneracy which allows them to anneal to

Table 1. *Applications of polymerase chain reaction (PCR) and reverse-transcription (RT)-PCR*

Technique	Application	References
PCR	Synthesis of labelled-DNA probes	Hirst *et al.* (1992)
	Molecular cloning	Delidow (1993)
	Sequencing DNA	Meltzer (1993)
	Preparation of mutant DNA sequences	Chen & Przybyla (1994)
	Screening transgenic bacteria and animals	Witsell & Shook (1993)
	Identification of sequence similarities	Pellegrino & Berg (1991); Preston (1993)
	Identification of relationships within population groups	Chen & Evans (1993)
	Screening fetal characteristics	Langlois & Wilson (1993)
RT-PCR	Detection of rare mRNA	Greco (1993); Witsell & Shook (1993)
	Survey of tissue or cell distribution of mRNA	Volkenandt *et al.* (1992); Kelley *et al.* (1993)
	Detection of variations in patterns of iso-protein expression	Bottema & Sommer (1993)
	Quantification of mRNA and pre-mRNA	Lipson & Baserga (1989); Volkenandt *et al.* (1992)
	Determination of mRNA and, hence, amino acid sequences	Meltzer (1993)

regions not identical but closely conserved within the family (Preston, 1993). The portions of the genes lying between these conserved regions can then be amplified and their structure determined. Thus, the genes for proteins with similar function, for example the 'zinc-finger' proteins (Pellegrino & Berg, 1991) or equivalent proteins from different species can be identified. It is also possible to use PCR to generate mutations and this can provide a useful tool for the elucidation of structure–function relationships (Chen & Przybyla, 1994).

The ability to amplify to usable levels DNA obtainable in only minute amounts from small blood samples means that it is feasible to assess the genetic profile of relatively large numbers of individuals (Chen & Evans, 1993). This should provide opportunities for the analysis of the influence of genetic factors on responses to nutrients and the relative contributions of genetics *v.* nutrition to the incidence of a range of conditions such as coronary disease and susceptibility to malignancy. The ability to analyse trace quantities of DNA has been used also to assess the genetic make up of a fetus during gestation by analysing the very small proportion of fetal cells circulating in maternal blood (Langlois & Wilson, 1993).

PCR is also extensively used in sequencing DNA, including cDNA, thus providing the amino acid sequences of the corresponding proteins (Meltzer, 1993). In contrast to most PCR reactions only a single primer is used for sequencing reactions, so that only the strand of interest is amplified and labelled before its analysis. The resulting amplification is linear with cycle number rather than logarithmic and the technique requires a higher initial DNA concentration than is commonly employed with standard PCR.

RT-PCR, transcription of RNA into DNA before its amplification by PCR, permits detection of mRNA as well as DNA (Volkenandt *et al.* 1992; Kelley *et al.* 1993). The sensitivity of this technique means that investigation of mRNA expression is possible even where the material available is extremely limited, as for example during studies of

embryonic development (Greco, 1993) or when screening cell lines during cloning (Witsell & Shook, 1993). It is also possible to investigate the primary product of transcription even though relatively rapid splicing ensures that it may be present at a concentration well below that of the corresponding mRNA (Lipson & Baserga, 1989). Furthermore, if the primers are carefully chosen to span regions of difference, it is often possible to distinguish closely-related mRNA such as those associated with isoenzymes or tissue-specific transporter proteins (Bottema & Sommer, 1993). As well as detecting the presence of specific mRNA, it is possible to obtain quantitative estimates, although the high degrees of amplification generally implicit in the use of PCR require special consideration in order to provide valid data (Volkenandt *et al.* 1992; see also below).

EXPERIMENTAL CONSIDERATIONS

Although previously recognized, PCR has only been widely applicable since thermostable DNA polymerases became available. A typical cycle of amplification involves heat denaturation at 95° to separate the strands, annealing at 40–60° and primer extension at 72° (Fig. 1). The denaturation step inactivated the polymerases initially employed, which then had to be added afresh during each cycle rendering the process laborious and expensive. However, currently-available thermostable polymerases, of which Taq was the first and still the most commonly used, are capable of retaining activity for up to thirty to forty cycles. This allows incubation mixtures containing all four deoxynucleotides, the two oligonucleotide primers and the polymerase to undergo multiple rounds of amplification in a programmable thermocycler without manual intervention.

The fundamental simplicity of the present methodology means that large numbers of samples can be processed in parallel but several factors must be considered for the results to be of value, one major consideration being the choice of primers. These oligonucleotides are now readily prepared by chemical synthesis. However, they must be chosen to form reasonably stable hybrids at the site of interest and their sequence should preclude binding to other sites within the template (Bloch, 1991; Erlich *et al.* 1991). Furthermore, they must not hybridize with the second PCR primer nor should they be liable to self-hybridize, since each of these conditions would result mainly in the production of short lengths of DNA containing primer dimers. Computer programs are now available to aid selection of suitable primers which are generally twenty to thirty nucleotides long. Success with PCR also depends on optimizing the Mg concentration in the reaction mixture which can influence both the extent and specificity of the reaction.

If the aim is simply to detect the presence of specific DNA or mRNA or to produce substantial quantities of a specific DNA, development of a satisfactory system is generally relatively straightforward. However, where quantitative results are important, considerable thought and technical expertise is often required. By the nature of PCR, amplification occurs logarithmically. Thus, small differences in amplification efficiency between samples and within samples between cycles will result in substantial variation in the yield of product. A variety of strategies have been developed to minimize these difficulties. Parallel amplification of standards is not generally satisfactory because of inter-sample variation and methods based on internal standardization are preferable (Volkenandt *et al.* 1992). These may involve amplification of a known amount of a second product using its own separate primers, but variations in efficiency of amplification between primers limit the usefulness of this technique (Khan *et al.* 1992). A more

satisfactory approach involves amplification of an internal standard with the same primers used with the experimental DNA (Van den Heuvel *et al.* 1993). In this case, however, the products of amplification of the experimental and standard DNA must be distinguishable generally by electrophoretic separation. The internal standard may be designed to yield a different length product, but possibly the most elegant approach is to use a version of the experimental sequence mutated such that it lacks or contains an extra restriction site (Gilliland *et al.* 1990). In this case, the experimental and standard templates may differ by only one or two bases and are amplified under identical conditions yet they can be separately estimated after electrophoresis of the restricted products.

PCR is increasingly utilized to detect and estimate mRNA by first reverse transcribing them into DNA. However, the complementary DNA (cDNA) so formed closely resemble sections of the chromosomal DNA and trace contamination of the RNA with the latter can totally invalidate the technique. Various strategies have been developed to minimize this problem. Thus, the two primers can be chosen to hybridize with different exons yielding products from genomic DNA containing intron sequences which can be separated by size from the mRNA products which lack these sequences (Chelly *et al.* 1990). Use is also commonly made of the poly A tail present in most mRNA but not in the genomic DNA to prime RT-PCR with oligonucleotides containing poly dT (Moore *et al.* 1990). Alternatively reverse transcription may be primed with a sequence specific at its 3′ end to the mRNA of interest but having a 5′ sequence not naturally present in the DNA (Shuldiner *et al.* 1991). The subsequent PCR reactions are then primed with sequences matching this unique 5′ sequence since these will only be present in the products of reverse transcription of mRNA.

CONCLUSIONS

The many variants of PCR provide an extremely powerful range of techniques for the investigation of biological samples but their very potential to amplify minute traces of DNA requires extreme care to be exercised in their execution and interpretation.

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