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The Polymerase Chain Reaction in Molecular and Micro-biology

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The polymerase chain reaction

The polymerase chain reaction (PCR) is an *in vitro* enzymatic method of amplifying specific DNA sequences conceived by Kary Mullis in 1983 (Mullis, 1990). The simple concept of the PCR relies upon the repeated synthesis of the targeted DNA by DNA polymerase. There are three steps needed to achieve this synthesis, which are carried out repeatedly and in succession at different and controlled temperatures, allowing for the doubling of the amount of DNA after each cycle (*Figure 1*). These three steps are:

1. Denaturation—a high temperature incubation (90–98°C) which melts the double-stranded DNA containing the target region into separate strands.
2. Annealing—a lowering of the temperature (typically to 37–65°C) in order to allow two synthetic oligodeoxyribonucletides (primers) to anneal to sites flanking the region of DNA to be amplified. Primers are added to the reaction mixture in a vast molar excess to favour formation of the primer–target complex rather than renaturation of the target DNA. The primers anneal to opposite strands of the DNA with their 3′ ends facing each other.
3. Extension—alteration of the temperature to the optimum for the DNA polymerase allows abstraction of deoxynucleotides (dNTPs) from the reaction mixture for the 5′ to 3′ synthesis of DNA directed from the primers, using the target DNA as template. Through this process the primers become incorporated into the PCR product.

Abbreviations: AP-PCR, arbitrary primer PCR; 2, 3-D, 2,4-dichlorophenoxyacetic acid; DMSO, dimethyl sulphoxide; dNTP, deoxynucleotide; EtBr, ethidium bromide; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; PEG, polyethylene glycol; RAPD, randomly amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; Taq, *Thermus aquaticus*; TMAC, tetramethyl ammonium chloride; Tth, *Thermus thermophilus*.

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Initially the Klenow fragment of *Escherichia coli* DNA polymerase I was used with an extension temperature of 37°C (Saiki *et al.*, 1985), however the thermal lability of the enzyme necessitated the addition of fresh enzyme after each denaturation step. The use of DNA polymerase from the extreme thermophilic bacterium *Thermus aquaticus* (Taq DNA polymerase), however, obviates the need to replenish the enzyme at each cycle (Saiki *et al.*, 1988) and makes the execution of the PCR more amenable to automation.

Each cycle of the PCR in theory results in a doubling of the target sequence flanked by the primers, as the extension products of each cycle are complementary to and capable of binding the primers in further synthesis cycles. Two species of amplification product are formed: the short product, which is the desired product (defined as the region between the 5′ ends of the primers, with discrete ends corresponding to the primer sequences); and the long product, derived from extension of primers annealed to the original DNA sample, which have a variable 3′ end. In theory an exponential accumulation of short product is achieved, while the long product accumulates arithmetically. A typical PCR occurs over 25–30 cycles, by the end of which the short product is overwhelmingly abundant (*Figure 1*).

SPECIFICITY OF THE PCR

The specificity of the PCR essentially depends upon the initial interaction of the primers with the DNA template. Although the primers used in PCRs are typically 20 or more nucleotides long, thus allowing for stringent (high temperature) annealing, any interaction of primer with genomic template may give rise to an extension product; indeed, it has been observed that primers with homology of only two base pairs (bp) can direct synthesis of target genes using annealing temperatures of 37°C (Sommer and Tautz, 1989). What makes the amplification of a defined target feasible is the dependence of the reaction on the specificity of *two* primers, which *must* bind to sites on either complementary strand of DNA. A high degree of specificity is only required during the early cycles of the PCR as during the later cycles most of the templates are the 'perfect' targets amplified in previous cycles (Ruano, Brash and Kidd, 1991). Obviously, if heat-labile DNA polymerases were to be used for PCR, the great specificity of primer–+template interactions obtained by high temperature annealing would not be possible. Thus the advent of thermostable enzymes such as Taq DNA polymerase has not only allowed for simple execution and automation of the PCR, but has imparted an increase in specificity for the target to be amplified.

Increasing the specificity of PCR

Although the most important factors governing the specific annealing of primers to template are the length and temperature of the annealing step (Wu *et al.*, 1991), which should be optimized for each primer pair (Rychlik,

Spencer and Rhoads, 1990; Wittwer and Garling, 1991), there have been many reports of variations on basic PCR methodology purported to increase priming specificity. The usual reaction mix used for PCRs with Taq DNA polymerase contains 50 mM potassium chloride (KCl), 10 mM Tris-HCl, 1·5 mM magnesium chloride ($MgCl_2$) and 200 μM of each dNTP; this reaction mix has been successful for many amplifications, although where PCR amplification is not possible the reason is frequently an inappropriate concentration of magnesium ions (Mg^{2+}). Because free magnesium is an absolute requirement for Taq DNA polymerase activity, the binding of Mg^{2+} by DNA, primers, dNTPs or chelators such as EDTA in stock reagents may significantly decrease the concentration of free magnesium ions and so prevent primer extension by the DNA polymerase (Innis and Gelfand, 1990). There may therefore be a temptation to increase the magnesium concentration in the stock PCR buffer to alleviate this problem. However, high concentrations of Mg^{2+} can lower the specificity of primer–template interactions and increase the possibility of mis-priming and the production of 'primer-dimers', where the primers use each other as template and produce a large amount of low molecular weight product (Williams, 1989a). In order to avoid wasting time over the tedious task of optimizing magnesium concentration, organic solvents such as formamide (Comey, Jung and Budowle, 1991), glycerol, polyethylene glycol (PEG), or dimethyl sulphoxide (DMSO) can be used successfully to facilitate the PCRs (Pomp and Medrano, 1991). Although these compounds may assist in the amplification of specific products, at concentrations above about 5% (w/w) they may significantly inhibit the activity of Taq DNA polymerase, thus reducing the amount of PCR product obtained (Gelfand and White, 1990). Tetramethylammonium chloride (TMAC) has also been used to increase the specificity of PCRs, but without the associated problems of inhibition of Taq DNA polymerase (Hung, Mak and Fong, 1990).

A very simple way to increase the specificity of the first cycle of the PCR is to exclude an essential reagent from the reaction until the mixture has reached a temperature of 70°C or more, the so-called *hot-start*. By addition of, for example, Taq DNA polymerase or dNTPs after heating the sample, we avoid the extension of primers which have annealed non-specifically to template DNA at the low temperatures of the laboratory bench or even the ice-bucket. As Taq DNA polymerase has significant activity between 37 and 50°C (Gelfand and White, 1990), these mismatched primers may be extended during the initial heating of the sample and remain in the reaction to give non-specific 'ugly little fragments' (Mullis, 1991), which may subsequently be amplified and significantly reduce the yield of the desired PCR product. Hot-start PCR protocols have been shown to remove most of the non-specific/undesired products typically produced in 'cold-start' protocols and should be widely adopted. The actual execution of 'hot-start' may be greatly simplified, particularly when multiple samples are being processed, by using a wax overlay for the reaction with an essential reagent lying above the wax barrier. On heating the wax melts and the final reagent is mixed convectively with the rest of the reaction (Chou *et al.*, 1992).

FIDELITY OF THE PCR

For many applications of the PCR random errors produced during primer extension are of little consequence, for instance where characterization of the PCR product by presence on an agarose gel or by nucleic acid hybridization is all that is required. However, if the PCR is being utilized in order to study allelic polymorphism or the amplification of genes for cloning to produce a functional protein, then the fidelity of the PCR is of paramount importance. Factors which may affect the error rate during PCRs are: DNA damage such as deamination of cytosine to produce uracil during high temperature incubations, strand-switching or 'jumping PCR' between templates when damaged or ancient DNA is used as template (Paabo, Irwin and Wilson, 1990), and the innate fidelity of the DNA polymerase utilized. The thermal damage to DNA and strand-switching are largely unavoidable, but careful selection of PCR parameters and DNA polymerase may help to minimize replication errors.

High-fidelity replication with Taq DNA polymerase

The most common enzyme used in PCRs, namely Taq DNA polymerase, lacks any associated 3′ to 5′ exonuclease activity for proof reading (Tindall and Kunkel, 1988). In the initial work using Taq DNA polymerase in PCRs an error rate of 1 base per 8000 incorporated was quoted (Saiki *et al.*, 1988). This order of base misincorporation frequency has also been found in subsequent studies (Dunning, Talmud and Humphries, 1988; Higuchi, Krummel and Saiki, 1988; Krawzcak *et al.*, 1989; Tindall and Kunkel, 1988; Fuchareon *et al.*, 1989; Ho *et al.*, 1989; Horton *et al.*, 1989; Myers, Sheffield and Cox, 1989; Jones and Howard, 1990). Some studies have indicated that the error frequency may be adjusted by alteration of various PCR parameters. During a study into the use of Taq DNA polymerase in DNA sequencing, Innis *et al.* (1988) found that low concentrations of dNTPs, or when the concentration of one dNTP was low relative to the others, a higher level of base misincorporation was seen. Also in 1988, Tindall and Kunkel demonstrated an increase in error frequency with increase in temperature. In 1990 Eckert and Kunkel ascertained the conditions necessary for high fidelity DNA synthesis by Taq DNA polymerase. They discovered that mutation frequency increased with increasing magnesium chloride concentration, with best fidelity being found when the concentration of magnesium chloride was equimolar with the dNTPs. High fidelity was also associated with low pH (pH 5–6) during primer extension and low (1 μM) dNTP concentration. Other studies have also given reaction constituent concentrations and thermal cycling conditions purported to improve the fidelity of the PCR with Taq DNA polymerase (Saiki *et al.*, 1988; Goodenow *et al.*, 1989; Myers, Sheffield and Cox, 1989; Williams, 1989a). However, despite these studies, there appears to be little concordance between research groups as to optimal conditions for fidelity. Indeed, it appears that conditions for optimal fidelity may vary between individual PCR reactions, different thermal cycling equip-

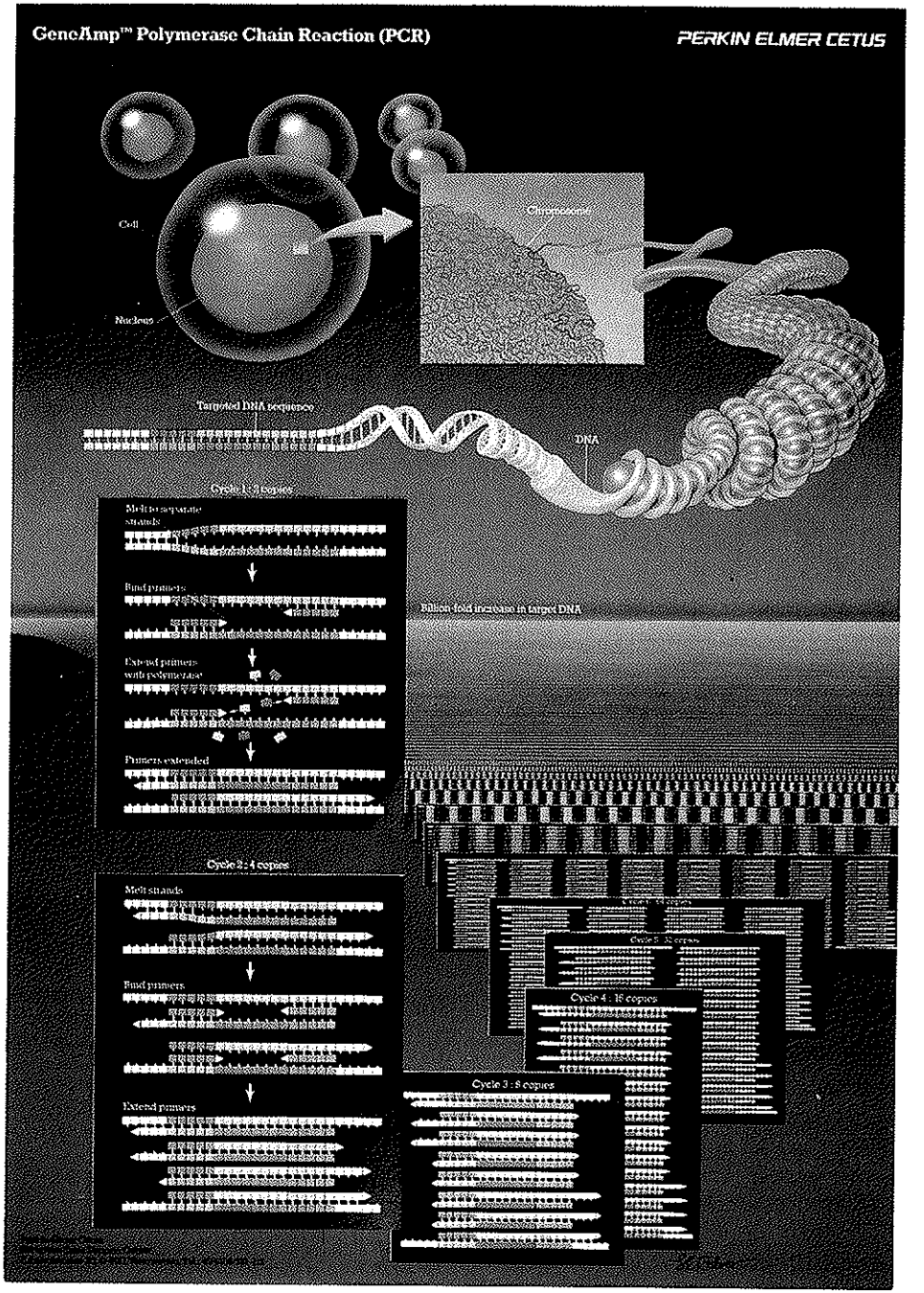

Figure 1. A diagrammatic representation of the principle of the polymerase chain reaction by Lewis B. Calver. Reproduced with the kind permission of Perkin Elmer Cetus.

ment and between laboratories (Chen and Viola, 1991; P.J. Hill and S. Swift, unpublished observations).

Alternative enzymes for PCR

Given that thermostable DNA polymerases are the enzymes of choice for the PCR, it is not surprising that there has been a proliferation of such enzymes available on the market. Some new products, such as Perkin Elmer Cetus's Amplitaq® Stoffel fragment, are genetically modified versions of well-documented enzymes such as Taq DNA polymerase, which give improved thermostability. Other enzymes such as those from *Thermus thermophilus* (Tth DNA polymerase; Myers and Gelfand, 1991a) and *Thermus flavis* (Replinase, Tet-Z polymerase) give greater thermal stability than Taq DNA polymerase but have a similar error rate in the PCR (Eckert and Kunkel, 1991). These enzymes, however, have the added advantage of significant reverse transcriptase activity, facilitating the amplification of DNA by using the PCR from RNA templates (Myers and Gelfand, 1991b).

Another group of newly available DNA polymerases from extreme thermophiles exhibit 3′–5′ proof reading activity and thus as much less error prone during primer extension. These, therefore, are the enzymes of choice where high-fidelity PCR is imperative. Another benefit of the 3′–5′ exonuclease activity is that the final PCR product possesses blunt ends that facilitate the cloning of PCR-derived DNA. This group of proof-reading enzymes includes: Pfu polymerase from *Pyrococcus furiosus* (Bergseid *et al.*, 1991; Lundberg *et al.*, 1991; Mathur *et al.*, 1992), Vent™ polymerase from *Thermococcus litoralis* (Cariello, Swenberg and Skopek, 1991; Ling *et al.*, 1991; Mattila *et al.*, 1991; Lohff and Cease, 1992) and Deep Vent™ polymerase from *Pyrococcus* sp. GB-D.

CONTAMINATION

The greatest strength of the PCR, namely the ability to amplify even a single DNA molecule is also its Achilles heel. Even minute quantities of extraneous DNA finding their way into the PCR reaction may serve as a PCR template and lead to confusing results and/or false positives; a major drawback if the PCR is being used for the diagnosis of genetic diseases or the detection of pathogens (Lo, Mehal and Fleming, 1988). In order to avoid such contamination, steps which can be taken may include; use of positive displacement pipettes and non-reusable containers, wearing gloves, aliquoting PCR mixes in sterile hoods, keeping ultra-clean stock solutions, the physical separation of pre- and post-PCR steps and the judicious use of control reactions (Bell, 1989). The use of autoclaved material is unacceptable as the autoclaving process does not destroy contaminating DNA, a fact which has been taken advantage of for safety reasons in the amplification of DNA from highly infectious micro-organisms (Barry and Gannon, 1991).

Apart from taking extreme care over the setting up of PCR mixes, a number of other techniques have been developed to remove contaminating

material from reactions. These techniques include restriction enzyme digestion (DeFilippes, 1991), DNAase treatment (Furrer *et al.*, 1990) and irradiation with ultraviolet light (Jinno, Yoshiura and Niikawa, 1990; Sarkar and Sommer, 1990) or γ-radiation (Deragon *et al.*, 1990).

Microbiological applications of the PCR

The application of the PCR in microbiology has had profound effects upon the speed and sensitivity with which micro-organisms can be detected and identified. Ease of execution of the PCR and its ability to amplify specific genes has enabled the targeting of important pathogens within complex mixtures of organisms. This, coupled with the sensitivity inherent in amplification of a single copy of the target gene has allowed detection times to be greatly reduced; lengthy enrichment and selective cultures are not necessary in order to obtain a detectable signal.

THE DETECTION OF MICRO-ORGANISMS IN FOOD

Food producers and processors must assess whether their products contain spoilage or pathogenic organisms before they are sent to retail outlets. However, there is often difficulty in detection and identification of particular pathogens within the mixed microbial population of the foodstuff. To this end many PCR-based detection systems have targeted the toxin genes of pathogenic strains so that only the potentially harmful bacteria are detected.

In early studies (Olive, Atta and Setti, 1988; Furrer, Candrian and Luthy, 1990; Johnson *et al.*, 1991) toxin gene sequences, identified in certain pathogenic *Escherichia coli* were used to specifically detect the enterotoxigenic strains. Subsequent studies have successfully detected and identified enterotoxigenic *E. coli* in mixed microbial populations within food and calf faecal samples (Samadpour *et al.*, 1990; Meyer, Lüthy and Candrian, 1991; Wernars *et al.*, 1991). The PCR has also allowed the specific detection of *Shigella flexneri* in food without the problems of distinguishing between *E. coli* and other *Shigella* spp. often encountered using more traditional biochemical identification techniques (Lampel *et al.*, 1990). The PCR has also been used for the detection of spoilage organisms in foods, such as the specific detection and identification of *Carnobacterium* spp. in the highly diverse bacterial flora of fresh meat (Brooks *et al.*, 1992).

While the PCR has proved to be successful for the detection of many food-borne micro-organisms, its great strength lies in its ability to detect and identify rapidly organisms which by current methods require lengthy enrichment and selection protocols. Prior to the PCR-based work of Szabo, Pemberton and Desmarchelier (1992), *Clostridium botulinum* was only identifiable using a bioassay based upon mouse lethality requiring a minimum of three days and often considerably longer. The highly pathogenic *Vibrio vulnificus* has also been successfully targeted by PCR techniques, both as culturable cells in raw oysters (Hill *et al.*, 1991) and as viable but non-

culturable cells, which are undetectable by traditional microbiological methods (Brauns, Hudson and Oliver, 1991).

Most studies concerning the PCR-based detection of food-borne bacteria have targeted *Listeria monocytogenes*, probably due to a perceived increase in food-borne listeriosis and a need to replace the time-consuming classical techniques for its detection and identification (Hayes *et al.*, 1991). A number of studies have identified specific DNA sequences in *L. monocytogenes* which do not show cross-reactivity with other *Listeria* spp. or other bacterial genera. These probes are based upon the sequences of genes coding for major secreted proteins (Flamm, Hinrichs and Thomashow, 1989), β-haemolysin (Datta, Wentz and Hill, 1987; Datta *et al.*, 1988), listeriolysin-O (Mengaud *et al.*, 1988), the delayed hypersensitivity factor (Notermans *et al.*, 1989), a listeriolysin-linked metalloprotease (Domann *et al.*, 1991) and cell surface proteins (Wang, Cao and Johnson, 1992), as well as randomly cloned *L. monocytogenes* DNA shown not to cross-react with other *Listeria* spp. (McKee *et al.*, 1991). Using PCR primers directed at these target genes a number of studies have succeeded in the specific detection of *L. monocytogenes* in pure bacterial cultures and in a number of foods (Bessesen *et al.*, 1990; Border *et al.*, 1990; Furrer *et al.*, 1991; Golsteyn-Thomas *et al.*, 1991; Bohnert *et al.*, 1992; Fitter, Heuzenroeder and Thomas, 1992; Neiderhauser *et al.*, 1992). Without exception, these studies have failed to detect the low detection limits required by public health authorities, although a recent innovation by Starbuck, Hill and Stewart (1992) has allowed the detection of *L. monocytogenes* in milk at levels below conventional plate-count methods. The detection limit of PCR-based techniques is, therefore, for the time being not limited by the PCR process *per se* but by the limitations of the procedures used to extract DNA from the target organisms; this problem is discussed further in the section *PCR sample preparation*.

THE DETECTION OF MICRO-ORGANISMS IN THE ENVIRONMENT

The detection of micro-organisms in the environment is both necessary for public health reasons and useful for the isolation of indigenous or introduced microbes with novel metabolic activities (Atlas *et al.*, 1992). Many of the problems of detecting micro-organisms in environmental samples are similar to those encountered when dealing with food samples, i.e. the need to identify particular species in a highly heterogeneous matrix with a highly mixed microbial flora, a process which is hindered by the need for lengthy enrichment and selective cultures prior to identification by biochemical, immunological or DNA hybridization techniques. it is therefore not surprising that the remarkable specificity and sensitivity of the PCR is beginning to be harnessed in the analysis of environmental samples.

Probably the most important environmental niche for micro-organisms from a public health standpoint is water. Lakes, rivers and other watercourses are regularly screened for the presence of pathogenic or indicator organisms, particularly where potable water supplies are involved. As water is also a relatively simple matrix from which to recover micro-organisms, a number of

studies have shown the specific detection of indicator coliforms in water using PCR-based probes. Bej *et al.* (1990a) showed that PCR primers for amplification of *E. coli lacZ* could detect all coliforms except Salmonellae, while PCR amplification of the *E. coli lamB* gene could specifically detect *E. coli, Salmonella* spp. and *Shigella* spp. in 100 ml water samples with detection limits as good as those obtained by conventional techniques. The utilization of both the *lacZ* primers and primers directed against the *uidA* gene of *E. coli* in the same PCR mixture (multiplex PCR), enabled the detection of coliforms and specifically *E. coli* in one reaction (Bej *et al.*, 1990b; Bej, McCarty and Atlas, 1991). Wide specificity primers based upon the highly conserved regions of the *luxA* gene have also enabled the PCR-based detection of marine luminous bacteria (Wimpee, Nadeau and Nealson, 1991).

Multiplex PCR has been used to target the highly fastidious pathogen *Legionella pneumophila*. Primers directed against the *Legionella* 5S rRNA genes allow the detection of any member of the genus whereas primers against the *mip* gene allow specific detection of pathogenic *L. pneumophila* (Bej, Mahbubani and Atlas, 1991). Multiplex PCR using these two primer pairs may be used to specifically detect *L. pneumophila* and/or the presence of other *Legionella* spp. (Bej *et al.*, 1990b) in water. Use of the PCR for the detection of micro-organisms in soils and sediments has also been demonstrated; for instance Josephson *et al.* (1991) demonstrated the presence of faecal coliforms in soil using a *lamB*-specific PCR primer pair.

Recombinant bacteria released into the environment have been targeted using the PCR. *Rhizobium leguminosarum* containing the transposon *Tn5* could be rapidly detected in low numbers in soil samples (Pillai *et al.*, 1991). Steffan and Atlas (1988) have demonstrated the detection of *Pseudomonas cepacia* (genetically engineered to degrade herbicides) in river sediments using the PCR, and Chaudhry, Toranzos and Bhatti (1989) detected *E. coli* in lake water which had been genetically engineered to degrade the herbicide 2,4-D (2,4 dichlorophenoxyacetic acid).

Although most environmental micro-organisms targeted for detection by the PCR have been bacteria, recently the successful detection of *Giardia* spp. by PCR has been reported (Mahbubani *et al.*, 1991a), opening the way for detection of other important protozoan pathogens such as *Cryptosporidium* spp.

THE PCR IN DIAGNOSIS

The area of science upon which the development of the PCR has had the most profound effect is without doubt medicine. Since its first use in the diagnosis of sickle cell anaemia (Saiki *et al.*, 1985) there have been hundreds of publications charting its use in the diagnosis of genetic disorders and cancers, applications beyond the scope of this review but previously reviewed by Reiss and Cooper (1990) and Lyons (1992). Many medical microbiology laboratories have also developed PCR-based techniques to assist in diagnostic microbiology, particularly for the detection of fastidious pathogens, viruses and parasitic protozoans.

The clinical microbiology laboratory is required to provide clinicians with information concerning the absence or presence of microbial pathogens as quickly and accurately as possible. The application of the PCR in disease diagnosis, therefore, has the potential to greatly increase the throughput of such laboratories by providing rapid detection and identification of pathogens without the need for culturing them, hence avoiding the associated time delays and biohazards of growing highly pathogenic organisms. Since early studies into the detection of food-poisoning bacteria in stool samples (Olive, 1989), many disease-causing organisms have become amenable to PCR detection. Those most often targeted are fastidious bacteria which are difficult to culture, viruses and protozoans. Examples of the detection of such pathogens are given in *Table 1*. Although all the examples given in *Table 1* are the causative agents of human disease, there are also numerous veterinary applications of the PCR for the detection of pathogens from livestock animals such as cows and pigs; for example, the detection of African swine fever virus (Steiger *et al.*, 1992), bovine viral diarrhoea virus (Ward and Misra, 1991) and bovine leukaemia virus (Sherman *et al.*, 1992). The PCR is also beginning to make a significant impact upon the diagnosis of plant disease, allowing the detection and differentiation of fungal pathogens such as *Verticillium* spp. (Nazar *et al.*, 1991) and non-culturable pathogens like viroids, such as citrus exocortis viroid (Rigden and Rezaian, 1992).

THE PCR IN MICROBIAL TAXONOMY

As well as applications in the detection of micro-organisms, the PCR offers unique techniques for the differentiation and typing of closely related species. Prior to the introduction of PCR-based techniques the most commonly used method to type strains on the basis of their DNA content was based upon the total digestion of genomic DNA, followed by probing the digest with a radioactive probe. The probe reveals hybridizing fragments of varying size and this 'DNA fingerprint' can be related to the strain. This approach to typing is called restriction fragment length polymorphism (RFLP) analysis (Jeffreys, Wilson and Thein, 1985) and has been used extensively for genetic studies. (An alternative approach for bacteria based on plasmid profiling has previously been reviewed; Dodd, Stewart and Waites, 1990.)

A modification of the basic PCR technique such that one or two arbitrary primers are included in the reaction rather than two specific primers has been used to produce 'DNA fingerprints' based upon DNA amplification rather than upon DNA digestion. After a few cycles of low stringency PCR (low anneal temperature) followed by a number of high stringency PCR cycles (high anneal temperature), a discrete and reproducible set of products are found for each species. This technique has variously been called arbitrary primer PCR (AP-PCR; Welsh and McClelland, 1990) or randomly amplified polymorphic DNA (RAPD; Williams *et al.*, 1990), and since its conception has been successfully used for the taxonomy of bacteria such as *Staphylococcus* spp., *Streptococcus* spp. (Welsh and McClelland, 1990, 1991; Poddar and McClelland, 1992), *Rhizobium meliloti* (DeBruijn, 1992) and *Frankia* spp.

Table 1. Detection of human pathogens using the PCR

Pathogen	Associated disease	Reference
Borrelia spp.	Lymes disease and American relapsing fever	Picken (1992)
Brucella spp.	Brucellosis	Fekete *et al.* (1990)
Chlamydia trachomatis	Lymphogranuloma venerum, trachoma	Palmer *et al.* (1991), Gilroy, Thomas and Taylor-Robinson (1992)
Clostridium difficile	Colitis/diarrhoea	Wren, Clayton and Tabaqchali (1990)
Corynebacterium diphtheriae	Diphtheria	Pallen (1991)
Helicobacter pylori	Gastritis, peptic and duodenal ulcers	Clayton *et al.* (1992) Hammar *et al.* (1992)
Mycobacterium leprae	Leprosy	Hartskeerl, DeWit and Klatser (1989)
Mycobacterium tuberculosis	Tuberculosis	Cousins *et al.* (1992), Cormican *et al.* (1992)
Staphylococcus aureus	Toxic shock syndrome	Predari, Ligozzi and Fontana (1991), Tokue *et al.* (1992)
Treponema pallidum	Syphilis	Hay *et al.* (1990)
Leishmania spp.	Leishmaniasis	DeBruijn (1988)
Toxoplasma gondii	Toxoplasmosis	Savva *et al.* (1990)
Hepatitis C virus	Hepatitis	Bukh, Purcell and Miller (1992)
HIV	AIDS	Derossi *et al.* (1988), Muul (1990), Kellog and Kwok (1990), Brandt *et al.* (1992) and others
HTLV-I	T-cell leukaemia	DeBarun and Srinivasan (1989)
Cytomegalovirus	Various	Olive, Simsek and Al-Mufti (1989), Olive *et al.* (1989), Buffone *et al.* (1991)
Trypanosoma spp.	Trypanosomiasis	Moser *et al.* (1989)
Enteroviruses	Various	Zoll *et al.* (1992)
Human papilloma virus	Cervical cancer	Bauer *et al.* (1991), Czegledy *et al.* (1992) and others

(Simonet *et al.*, 1991; Sellstedt *et al.*, 1992), non-culturable mycoplasmas (Deng and Hiruki, 1991) and of fungi such as *Neurospora crassa* (Williams *et al.*, 1990) and *Fusarium* spp. (Crowhurst *et al.*, 1991). In addition to these studies, an amalgamation of RFLP and RAPD techniques (either by restriction digestion of PCR products or PCR of restricted DNA) has assisted in the taxonomy of a number of bacterial species including *Clostridium* spp. (Gurtler, Wilson and Mayall, 1991), *Brucella* spp. (Herman and DeRidder, 1992) and *L. monocytogenes* (Vines *et al.*, 1992). The major advantages of RAPD methods over RFLP methods are the reduced necessity for technical expertise, radioactive isotopes and large amounts of high purity DNA as starting materials.

LIMITATIONS OF THE PCR FOR MICROBIAL DETECTION

Although the ability of the PCR to amplify very low numbers of specific target molecules successfully is beyond doubt, there are still limitations which must be addressed before PCR-based techniques are widely accepted for microbial detection and diagnosis. Among these limitations are the needs for methods for the rapid and reliable extraction of target nucleic acid from complex samples, 'low-tech' alternatives for the analysis of PCR products and for quantification of target organisms, and methods to ensure that PCR products are derived from viable rather than dead organisms or free DNA in samples. The problem of contamination of PCRs may be overcome by good laboratory practice, as discussed in the section above on *Contamination*.

PCR sample preparation

A major limitation of PCR diagnosis is that many bodily fluids and products such as blood, urine, mucus, sperm and faeces have been shown to inhibit Taq DNA polymerase (Panaccio and Lew, 1991). Therefore pre-PCR protocols are required to remove inhibitors from the nucleic acid samples, protocols which are not required when attempting to amplify DNA from pure cultures of bacteria (Nishikawa, Fowlkes and Kay, 1989, Ross, Woodley and Baird, 1989; Sandhu, Precup and Kline, 1989; Joshi, Baichwal and Ames, 1991) or yeasts (Kwiatkowski *et al.*, 1990; Sathe *et al.*, 1991). Many preparative methods for nucleic acids are available and widely described in laboratory manuals and other such literature, but most are for the purpose of preparing large amounts of very 'clean' DNA by the use, for example, of caesium chloride gradients or phenol-chloroform extraction. For the PCR, however, only small amounts of DNA are needed, and this may be relatively unpurified. It is for this reason that many protocols have been based upon the use of crude lysates in the PCR mix. These crude lysate methods have been successfully used to give good PCR amplification from RNA and DNA and typically have protocols based upon cell lysis by either proteinase K digestion (Erlich-Kautzky, Shinomiya and Marsh, 1991; Hanley and Merlie, 1991; Weizsacker *et al.*, 1991), thermophilic protease digestion (Fung and Fung, 1991; McHale, Stapleton and Bergquist, 1991) or simply by boiling (Ferre and Garduno, 1989; Lin-Chao, Brenner and Cohen, 1990; Nordvag, Husby and El-Gewely, 1992). In addition alkaline lysis procedures originally designed for bacteria have been successfully used to recover viral DNA from mammalian cells (Lutze and Winegar, 1990) and direct amplication from small quantities of blood has also been successfully achieved (Mercier *et al.*, 1990). In an alternative approach, Panaccio and Lew (1991) discovered that Tth DNA polymerase is not as inhibited by whole blood as Taq DNA polymerase, although whether this enzyme is inhibited by other biological fluids is as yet unknown. A number of methods for nucleic acid preparation from fresh and paraffin-embedded tissues have previously been reviewed (Kawasaki, 1990; Wright and Manos, 1990).

Isolation of nucleic acids from environmental samples presents more

problems than clinical specimens, although rapid protocols are continuously evolving. The easiest environmental matrix from which to isolate nucleic acids is water. Cells may be recovered from relatively large volumes of water by simple centrifugation (Atlas and Bej, 1990); however, this is a slow and labour-intensive approach, particularly if many samples are to be analysed, and filtration is therefore the preferred alternative. If the water sample is filtered through a small diameter filter, the filter may be placed directly in an Eppendorf tube and the PCR carried out with the filter present, although many materials from which filters are manufactured appear to be inhibitory to Taq DNA polymerase (Bej *et al.*, 1991). Although the use of alternative thermostable enzymes such as Tth DNA polymerase may circumvent this problem (Skryabin *et al.*, 1990), the lower efficiency of the PCR and the necessity for extra cycles (Kadokami and Lewis, 1990) increases the chance of amplifying non-specific products (Bell and DeMarini, 1991). The removal of the filter matrix without loss of sample organisms is therefore desirable and to this end Starbuck, Hill and Stewart (1992) have recently developed a protocol by which the filter is totally solubilized, thus releasing *all* sample DNA for analysis. [Somerville *et al.* (1989) reported cell lysis and DNA recovery from a cylindrical filter membrane but DNA yields by this method were low].

The extraction of target nucleic acids from soil and sediment samples presents novel problems as even trace amounts of humic substances can inhibit the PCR and give false-negative results (Tsai and Olson, 1992a). Two approaches to this problem have been explored. One approach, exemplified by Pillai *et al.* (1991), involves the separation of target organisms from the soil samples on the basis of buoyant density, an approach which may lead to the loss of target organisms. The second approach is to lyse the target organisms *in situ* followed by purification of the DNA from the crude lysate. In a recent study Tsai and Olson (1992b) used such a technique, with DNA purification by gel filtration with Sephadex G-200 giving a very high recovery of bacterial DNA. The recent upsurge in popularity of ion-exchange resins and cartridges for DNA purification will undoubtedly lead to a facilitation of such methods.

The analysis of PCR products

Following PCR amplification there is a need to analyse the products and ensure that the required product has been synthesized. The methods used to identify and analyse PCR products vary from detection of a size-specific band by agarose electrophoresis, with or without restriction endonuclease digestion, to more technically difficult and time-consuming hybridization methods such as Southern blots and dot blots. Such methods are labour-intensive, difficult to automate and have low throughput, and so have contributed to a reluctance to use PCR-based techniques in many diagnostic laboratories.

A number of methods have emerged in an attempt to increase the speed of PCR product analysis. Kai *et al.* (1991) showed that by using a third 'nested' primer in PCRs the desired products, if amplified, would give two size-specific bands after agarose electrophoresis, therefore increasing the confidence of specific product amplification without the need for hybridization or

restriction mapping. Another method necessitating a third primer was developed by Parker and Burmer (1991): following thermal cycling an aliquot of the PCR mix is subjected to one extra PCR cycle in the presence of a 'nested' end-labelled primer. The product of this reaction is electrophoresed, blotted onto a membrane and subjected to autoradiography for detection of the specific product.

Many methods have been developed which rely upon the use of primers modified to contain fluorescent dyes (Chehab and Kan, 1989), biotin (Sauviago *et al.*, 1990), peroxidases (Levenson and Chang, 1990) or DNA binding protein recognition sites (Triglia *et al.*, 1990). As PCR primers are incorporated into the PCR product the desired product will contain the primer's label. Such protocols may allow the immobilization of the PCR product on a solid matrix via one primer (Sauviago *et al.*, 1990; Triglia *et al.*, 1990), while the other primer may contain a label to facilitate detection (Sauviago *et al.*, 1990).

Alternatively, labels can be directly incorporated into the PCR product by performing the amplification in the presence of nucleotides labelled with dioxygenin (Lanzillo, 1990; P.J. Hill, unpublished results) or biotin (Day *et al.*, 1990; Lo, Mehal and Fleming, 1990). Such labelled products may be directly detected following electrophoretic separation, high performance liquid chromatography (HPLC), or capture by sequence-specific probes on a solid matrix (Syvanen *et al.*, 1988; Saiki *et al.*, 1989).

All these methods require post-PCR handling of the amplified product, which may increase the chances of contamination. In order to avoid such problems Higuchi *et al.* (1992) have developed a method utilizing ethidium bromide (EtBr) by which successful amplification can be detected on the basis of the enhanced fluorescence of EtBr when it is bound to double-stranded DNA, without opening the PCR tube. A problem with this protocol involves the innate danger associated with EtBr; the development of other, less hazardous, DNA binding dyes which exhibit enhanced fluorescence upon binding double-stranded DNA may increase the utility of such an approach.

It may also be desirable to quantitate PCR products and thus to relate the amount of product to the number of original target sequences. Quantification may be carried out using affinity-based product collection as described by Syvanen *et al.* (1988), although errors may appear when sub-optimal PCR amplification occurs. In order to circumvent this problem a PCR quantitation system containing an internal standard is desirable. Such a system based upon a competitive PCR was reported by Gilliland *et al.* (1990), and relies upon the co-amplification of sample and competitor targets, the latter of which is present at different levels. The ratio of the products remains the same throughout the PCR and reflects the original target:competitor ratio. The final ratio of the PCR products can be readily quantitated following direct electrophoresis (if target and competitor DNAs are of different sizes) or eletgrophoresis following endonuclease digestion (if target and competitor DNAs are of similar size). A variation of this method allows target and competitor DNAs to differ by only a single base, thus avoiding differential synthesis rates of target and competitor fragments. The different PCR

products can be resolved using temperature gradient gel electrophoresis (Henco and Heibey, 1990).

Differentiation of live from dead organisms

Although the PCR may be proved to be invaluable for the detection of clinically important pathogens and environmental organisms, it is possible for the technique to give false positives due to amplification of DNA from dead organisms. This information may be of great importance where for instance the presence of toxigenic bacteria in raw food materials has led to the persistence of toxins in retail foodstuffs. Even so, in many instances, particularly diagnostic scenarios, the specific detection of live pathogens alone is desirable. The most common approach to this problem is to detect messenger RNA (mRNA), a nucleic acid which typically has a very short half-life. While the PCR is a DNA amplification technique, it may equally be applied to RNA detection if the RNA template is first reverse transcribed to produce cDNA (which is an effective PCR template), a process often referred to as RT-PCR. Many examples of reverse transcription to produce PCR templates are published (for example: Kawasaki, 1989; Ohara, Dorit and Gilbert, 1989; Kawasaki, 1990; Moore, Shepherd and Hoskins, 1990), often using reverse transcriptases originating from retroviruses. Such enzymes may inhibit Taq DNA polymerase activity (Sellner, Coelen and MacKenzie, 1992) and therefore necessitate extra sample manipulations between reverse transcription and the PCR. This increases the throughput time and the chances of contamination. Although Taq DNA polymerase possesses reverse transcriptase activity which may allow direct amplification of RNA in PCRs (Jones and Foulkes, 1989; Tse and Forget, 1990), this activity is not efficient enough for most applications. The recent appearance of alternative thermostable DNA polymerases with significant reverse transcriptase activity (see the section *Alternative enzymes for PCR*) will facilitate the use of such protocols. False positives resulting from contamination with minute quantities of DNA are possible with RT-PCR, although the frequency of false positives may be greatly reduced using techniques such as RS-PCR (Schuldiner, Scott and Roth, 1990) by which the cDNA is tagged with a unique sequence so that *only* cDNA is subsequently amplified, or by enzymatic digestion of contaminating DNA prior to reverse transcription and PCRs (Dilworth and McCarrey, 1992).

The use of direct PCRs coupled with RT-PCR has been demonstrated to differentiate successfully live from dead cells of bacteria such as *L. pneumophila* (Mahbubani *et al.*, 1991b; Bej, Mahbubani and Atlas, 1991) and protozoan pathogens such as *Giardia* spp. (Mahbubani *et al.*, 1991a).

ALTERNATIVE NUCLEIC ACID AMPLIFICATION SYSTEMS

Although the PCR was the first nucleic acid amplification technique to be developed and is the most widely recognized and utilized, there are now a number of alternative technologies for the amplification of nucleic acids.

These alternative technologies may differ in their template and enzyme requirements and many do not require expensive thermal cycling equipment as the amplification system is isothermal. A summary of some of these non-PCR amplification technologies is given in *Table 2*.

Table 2. Alternative nucleic acid amplification technologies

Technology	Template	Enzymes	Isothermal	Reference
Ligation chain reaction/ligation amplification reaction (LCR/LAR)	DNA	DNA ligase	No	Landegren *et al.* (1988), Wu and Wallace, (1989), Barringer *et al.* (1990), Barany (1991)
Strand displacement amplification (SDA)	DNA	DNA polymerase, Restriction endonuclease	Yes	Walker *et al.* (1992a, b)
Nucleic acid sequence-based amplification (NASBA)	DNA or RNA	Reverse transcriptase, RNA polymerase, RNAse H.	Yes	Kievits *et al.* (1991), Malek *et al.* (1992)
Self-sustained sequence replication (3SR)	RNA	Reverse transcriptase, RNA polymerase, RNAse H.	Yes	Guatelli *et al.* (1990), Fahy, Kwoh and Gingeras (1991)
Q-Beta amplification (Q-β)	RNA	Q-β-replicase	Yes	Lizardi *et al.* (1988), Lomeli *et al.* (1989), Kramer and Lizardi (1989)
Ligation activated transcription (LAT)	DNA	Ligase, RNA polymerase	Yes	Lewis (1992)
Boomerang DNA amplification (BDA)	DNA	DNA polymerase, Restriction endonuclease	Yes	Lewis (1992)
Repair chain reaction (RCR)	DNA	DNA polymerase, DNA ligase	?	Lewis (1992)
Sequence-independent single primer amplification (SISPA)	DNA	DNA ligase, DNA polymerase	No	Reyes and Kim (1991)

The use of the PCR in molecular biology

The profound impact of the PCR upon diagnostic sciences is mirrored in the field of molecular biology where, alongside techniques such as DNA sequencing and Southern blotting, it has become an indispensable tool in research laboratories. The utility of the PCR in molecular biology is not simply gained by the ability to generate large amounts of specific DNA within hours (traditional biological methods of DNA production may take a number of days), but also by the ability to alter DNA sequences via the PCR primers which become incorporated into the amplified product.

THE USE OF THE PCR IN CLONING

Traditional strategies for gene cloning invariably consist of the generation of a random genetic library in a bacteriophage or plasmid vector. This library is then screened for the desired clone by phenotypic expression in bacteria (usually *E. coli*) either directly or with antibodies, or is screened by hybridization with labelled DNA probes specific for the gene of interest. Such experiments are very time consuming and laborious. The PCR provides rapid alternatives to such protocols provided that some sequence information of the desired clone is available, either directly from previously sequenced genes or highly conserved regions of gene families, or indirectly as degenerate DNA sequences derived from the terminal sequencing of proteins.

A major concern when cloning DNA via PCRs is the fidelity of replication (see the section *Fidelity of the PCR*), as the cloned genes need to retain perfect identity with the wild-type gene. In 1991 Hill, Swift and Stewart showed that more than 83% of PCR-derived clones of both structural and regulatory genes retained full, wild-type, biological function, even when the PCRs were carried out under sub-optimal conditions (with respect to fidelity) with Taq DNA polymerase. *Figure 2* shows the basis of one of their assays, namely the retention of functionality of bacterial luciferase (*luxAB*) genes (cloned into *E. coli*) following 30 sub-optimal PCR cycles. Functionality (>83%) was expressed as percentage of light recombinant *E. coli* colonies relative to the number of colonies containing *lux* DNA as determined by colony hybridization. The availability of proof-reading thermostable DNA polymerases (see the section *Alternative enzymes for PCR*) should further increase faith in the fidelity of PCR derived clones.

Difficulties are frequently encountered when cloning PCR products directly as Taq DNA polymerase produces 'ragged ends' due to the non-templated addition of a single dAMP residue to the 3′ terminus of each DNA strand (Clark, 1988). A number of techniques have been developed to facilitate the cloning of PCR products. These include the 'polishing' of the PCR product's ends with the Klenow fragment of *E. coli* DNA polymerase I (Williams, 1989b; Lorens, 1991), the introduction of defined cohesive ends with T4 DNA polymerase (Stoker, 1990), the incorporation of restriction endonuclease recognition sites in the PCR primers and thus into the amplified product to facilitate directional cloning (Jung, Pestka and Pestka, 1990; Kaufman and Evans, 1990; Hill, Swift and Stewart, 1991), the construction of T-vectors containing single dTMP overhangs at their 3′ ends (Holton and Graham, 1991; Marchuk *et al.*, 1991; Kovalic, Kwak and Weisblum, 1991; Mead *et al.*, 1991), and the ligase-free cloning protocols which rely upon the production of gap–duplex recombinants between PCR product and plasmid vector (Aslanidis and DeJong, 1990; Schuldiner, Scott and Roth, 1990). The use of alternative proof-reading enzymes in PCRs will obviate such procedures, as the PCR products obtained by using such enzymes possess blunt ends and can thus be easily cloned.

PCR-based cloning protocols have successfully been used for the cloning of DNA directing the synthesis of functional genes from a number of species,

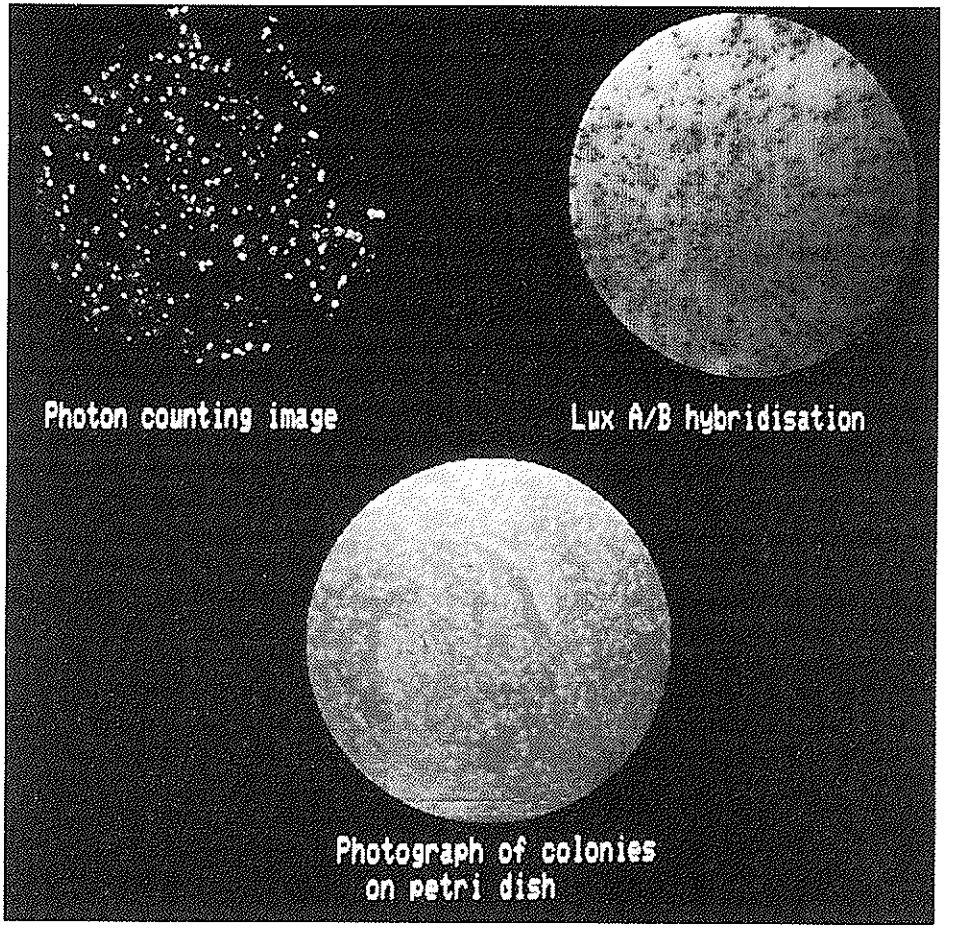

Figure 2. A direct comparison between PCR derived gene product functionality and gene presence. The presence of the *luxAB* genes was determined by filter hybridization against a [^{32}P] labelled *luxAB* probe (top right). LuxAB functionality was assessed by counting bioluminescent recombinant *E. coli* colonies (top left) following the addition of *n*-decanal vapour and using a Hammamatsu Argus 100 VIM 3 photon video camera. The ratios between total transformants (bottom centre), *lux*-containing transformants and bioluminescent transformants provides an excellent functional assay for PCR fidelity. Adapted from Hill, Swift and Stewart (1991).

including *Anabaena* (Fillat, Borrias and Weisbeek, 1991), *Clostridium* spp. (Nemoto *et al.*, 1991; Teller *et al.*, 1992), *E. coli* (Hill, Swift and Stewart, 1991; Patil and Dekker, 1992), *Streptococcus faecalis* (Ross and Claiborne, 1991) and *Vibrio harveyi* (Hill, Swift and Stewart, 1991). In order to facilitate the expression of recombinant DNA, PCR primers may contain (in addition to restriction endonuclease recognition sites) signal sequences such as promoters, ribosome binding sites and terminators. Introduction of such sequences has facilitated the high expression of PCR amplified DNA both *in vivo* (MacFerrin *et al.*, 1990; Slightom, 1991) and *in vitro* (Kain, Orlandi and Lanar, 1991).

As well as facilitating the cloning of genomic DNA, the employment of RT-PCR has enabled the rapid cloning of intron-less eukaryotic genes which retain their biological function (e.g. Akowitz and Manuelidis, 1989; Lerner and Shoemaker, 1992), which is preferable to the cloning of intron-containing genomic DNA or the PCR amplification of separate exons followed by their ligation to provide functional genes (Lebedenko *et al.*, 1991; Vandenbroeck *et al.*, 1991).

In addition to providing quick and simple methods for the cloning of genomic DNA and cDNA, the PCR provides rapid screening methods for cloned libraries either directly in bacteria or phage (Hovens and Wilks, 1989; Rapley and Walker, 1989; Schofield, 1990; Rosenberg *et al.*, 1991), or by using the PCR incorporating labels (either radioactive or non-radioactive) in order to rapidly produce DNA probes for hybridization-based screening (Levenson and Chang, 1990; Finckh, Lingenfelter and Myerson, 1991; Uchimura *et al.*, 1991).

THE USE OF THE PCR FOR ALTERATION OF DNA SEQUENCES

The introduction of deletions, insertions and point mutations in DNA sequences is a powerful approach to the analysis of the structure and function of genes and their products. Several techniques are available for site-directed mutagenesis, but these often require many steps and extensive screening to locate the mutant. As PCR primers become incorporated into the amplified DNA, mismatches may be included in the primers to give rapid site-directed mutagenesis (Mullis and Faloona, 1987; Dulau, Cheyron and Aigle, 1989; Kadowaki *et al.*, 1989; Tomic *et al.*, 1990; Hill, Swift and Stewart, 1991). The localization of these mutations at the end of the PCR product, however, limits this simplistic mutagenesis method. Other PCR protocols have been developed to enable site-directed mutagenesis in any region of a cloned gene. These rely upon the PCR to produce gap–duplex recombinant circles which can be used to transform *E. coli* directly (Jones and Howard, 1990, 1991; Jones and Winistorfer, 1992) or, more commonly, upon 'overlap extension', which is a purely PCR-based technique whereby one PCR product containing a mutation (point, deletion or insertion) is used as a primer in further PCR cycles to prime the synthesis of the remainder of the (unmutated) gene. This procedure results in 100% of the PCR product containing the desired mutation. A number of mutagenesis protocols based upon this 'overlap extension' or 'recombinant PCR' concept have been published (Higuchi, Krummel and Saiki, 1988; Ho *et al.*, 1989; Kammann *et al.*, 1989; Herlitze and Koenen, 1990; Perrin and Gilliland, 1990; Kuipers, Boot and Devos, 1991; Merino *et al.*, 1992).

The concept of overlap extension has also been successfully used for the engineering of fusion and hybrid genes (Yon and Fried, 1989; Horton *et al.*, 1989; Vallette *et al.*, 1989; Yolov and Shabarova, 1990) and for the construction of totally synthetic genes from oligodeoxynucleotides (Jayaraman, Shah and Fyles, 1989; Ciccarelli *et al.*, 1991).

In addition to primer-directed mutations during PCRs, the innate infidelity

of Taq DNA polymerase has been used to produce random point mutations (Leung, Chen and Goeddel, 1989). A number of successful studies have been conducted using this hitherto undesirable property of Taq DNA polymerase, for example, in the production of non-interactive mutants of tryptophan synthase subunits in *E. coli* (Swift, Kuhn and Stewart, 1992) and in the production of mutant mammalian secreted proteins (Rice *et al.*, 1992).

THE PCR AND DNA SEQUENCING

One highly significant use of the PCR is in the generation of DNA templates for sequencing. The basis for the utility of the PCR in sequencing is the ability to produce large amounts of specific DNA from a small amount of genomic template. This product may be sequenced directly using normal double-stranded sequencing protocols which obviate the need for cloning and the preparation of plasmid DNA. Linear double-stranded DNA may prove refractory to sequencing, however, because its tendency to re-anneal may prevent extension from the sequencing primer or prevent the sequencing primer from annealing (Bachmann, Luke and Hunsmann, 1990). Although sequencing problems with linear double-stranded DNA may be avoided by addition of single-stranded DNA with complementarity to one of the template strands (Gal and Hohn, 1989), a number of modifications of the PCR technique have been developed to facilitate the production of single-stranded templates which are more amenable to sequencing.

The first such protocol to be developed was asymmetric PCR (Gyllensten and Erlich, 1988; Innis *et al.*, 1988), which results in the production of single-stranded DNA directly from the PCR. This is achieved by using one of the primers at a limiting concentration, the limiting primer becomes exhausted after a number of cycles so that in further cycles extension only occurs from the non-limiting primer resulting in large amounts of single stranded DNA. This may be directly subjected to sequencing protocols. A number of variations of asymmetric PCR sequencing methods have been published (Shyamala and Ames, 1989; Wilson, Chen and Hood, 1990; Allard, Ellsworth and Honeycutt, 1991).

Other methods of producing single-stranded DNA sequencing template from PCR products rely upon the removal of one strand of the double-stranded product. This may be achieved using a number of methods, one of which relies upon the digestion of one strand using λ exonuclease. This only degrades strands of DNA having a 5′ terminal phosphate group which can be introduced into PCR products via *one* phosphorylated primer (Higuchi and Ochman, 1989). The other main method for specific strand removal relies upon the use of a biotinylated primer in the PCR. This allows one strand of the product to be bound to a solid support via a streptavidin–biotin link. The non-biotinylated strand is washed away following denaturation, leaving the attached single strand to be sequenced directly or after elution from the support (Kretz, Carsrien, 1989; Mitchell and Merril, 1989; Green, Roopra and Vaudin, 1990; Wahlberg *et al.*, 1990; Hultman *et al.*, 1991; Lagerstrom *et al.*, 1991).

A different approach provides a single-stranded RNA template which can be sequenced using reverse transcriptase. In order to produce this, a normal PCR is carried out with one primer having a phage promoter sequence at its 5′ end. *In vitro* transcription of the PCR product then gives a further amplification of the desired single-stranded RNA sequencing template (Stoflet *et al.*, 1988).

A purely PCR-based sequencing strategy is also available, consisting of a number of PCR cycles with a single primer in the presence of dideoxy nucleotides, which in effect provides a series of dideoxy termination sequencing reactions. The product is labelled with either ^{32}P or fluorescent labels on the primer, or by direct incorporation of ^{35}S dATP, to enable visualization of the bands following electrophoresis (Kusukawa *et al.*, 1990; Rohan, King and Frels, 1990; Craxton, 1991; Krishnan, Blakesley and Berg, 1991; Lee, 1991; Rao and Saunders, 1992). A number of PCR-based sequencing protocols have recently been published in a single volume (Ellingboe and Gyllensten, 1992).

OTHER USES OF THE PCR IN MOLECULAR BIOLOGY

The use of the PCR for cloning, mutation, gene fusion and sequencing as described above, although highly significant, only scratches the surface of the many exploitations of this powerful technique to simplify or speed up molecular biological methods including: S1 protection assays (Blakely and Carman, 1991), DNaseI footprinting (Tanguay, Pfeifer and Riggs, 1990), DNA–protein binding studies (Higuchi, Krummel and Saiki, 1988; Kinzler and Vogelstein, 1989; Pollock and Treisman, 1990; Rudert and Trucco, 1990), genomic walking (Fors, Saavedra and Hood, 1990; Rosenthal and Jones, 1990; Arnold and Hodgson, 1991; Parks, Chang and Shenk, 1991) and assaying gene expression (Mutchler, Klemish and Russo, 1992; Sperisen *et al.*, 1992). The potential of the PCR in research applications appears to be limited only by the imagination of research scientists, and novel methods are under continuous evolution.

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