15 Future Directions for PCR in Mycology

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15.1 Introduction

Polymerase chain reaction (PCR)-based amplification of target nucleic acids was conceived by Kary Mullis in California in 1983 (Mullis, 1990). To date it is estimated that since the first publication in 1985 there have been over 40,000 references to PCR (White, 1996). Over the last decade PCR has become one of the most important and powerful tools in molecular biology. Two crucial technological developments – the introduction of thermostable DNA polymerases and automated thermal cyclers – have led to the common use of PCR for research and diagnostic purposes. Considerable effort is underway to make the PCR technology more robust, cost effective and user friendly.

In this chapter, some of the recent developments in amplification and detection technologies, the currently available diagnostic PCR kits, future applications of PCR in mycology and perspectives are discussed. A number of PCR based applications (Table 15.1) are currently used routinely by many laboratories and will not be covered in detail here. Further information on these techniques can be obtained from other reviews (Erlich and Arnheim, 1992; Foster *et al*., 1993).

15.2 Molecular Ecology, Epidemiology and Strain Typing

Integration of genetic markers with biological traits has helped to address questions on mating systems, origin of strains, gene flow, and host–parasite and symbiotic interactions. RFLP-based markers have been used extensively

 Applications of PCR in Mycology (eds P.D. Bridge, D.K. Arora, C.A. Reddy and R.P. Elander)

Table 15.1. Present applications of PCR in mycology.

RAPD–PCR
RT–PCR
Inverse PCR
PCR mutagenesis
PCR gene construction
PCR SOEing*

*Splicing by overlap extension.

for this purpose. Recently, PCR technology has enabled rapid strides to be taken in this area. PCR-based multilocus genotyping is likely to resolve questions such as the enhanced virulence or transmissibility of strains and also the complexity of the pathogen population of a disease outbreak. For example, multilocus genotyping (arbitrarily primed PCR) of the asexual human fungal pathogen *Coccidioides immitis* revealed that the haploid organism was completely recombining, within geographically isolated populations, even though no sexual stage had been recorded (White, 1996). Such data can be used to trace the geographic origin of an infection/pathogen. In agricultural ecosystems such high resolution genetic information will be important to determine whether sexual recombination and gene flow are occurring regularly during a pathogen's life history. Reassortment of the virulence genes, leading to new combinations, could very quickly overcome genes introduced for resistance. PCR is also likely to negate the limitations imposed on our understanding of microbial diversity using traditional microbiological methods. A number of PCR-based techniques suitable for these applications are emerging.

15.2.1 Amplified fragment length polymorphism (AFLP) analysis

AFLP is a tool that allows differentiation between individuals, genotypes and strains and the assessment of genetic diversity and phylogeny. Originally developed for genetic mapping in plants (Vos *et al.,* 1995), this technique has the characteristics of an ideal system for detecting genetic variation.

The AFLP markers generated are 'neutral' (i.e. not subject to natural selection) and are generated from a large number of independent loci from different parts of the genome. The profiles generated are reported to be highly reproducible, because of the high stringency PCR due to the nature of the primers (Majer *et al.,* 1996; Mueller *et al.,* 1996).

AFLP analysis involves selective amplification of fragments from restriction enzyme digests of genomic DNA. Approximately 500 ng of genomic DNA is digested simultaneously with a hexanucleotide-cutter *Eco*RI and a tetranucleotide-cutter *Mse*I. This results in more than 150,000 fragments from a fungal genome of approximately 5×10^7 bp. The digested

fragments are ligated with an *Mse*I adaptor and a biotinylated *Eco*RI adaptor. Fragments with the *Eco*RI adaptor are captured using streptavidin beads for PCR. The numerous, small *Mse–Mse* fragments are discarded. Primers are designed to match each of the restriction enzyme adaptor sequences with the addition of an arbitrary two base extension at the 3′ ends and PCR is then performed at high stringency (Fig. 15.1). This leads to the amplification of a manageable number of fragments. Usually 50–70 fragments can be resolved from one PCR on denaturing polyacrylamide gels and the whole procedure can be completed in 2 days. Ready-to-use kits (e.g. Life Technologies, Perkin Elmer/Applied Biosystems) are available for AFLP analysis.

15.2.2 Long PCR

Restriction fragment length polymorphisms (RFLPs) in rDNA and mtDNA have been used extensively in assessing genetic diversity and species relationships in various fungi. Conventionally, polymorphisms are detected by Southern hybridization of the digested genomic DNA samples with a specific probe (e.g. Sreenivasaprasad *et al*., 1992). RFLPs from PCR amplified segments (up to 2.0–2.5 kb) of rDNA have also been used in similar studies (e.g. Muthumeenakshi, 1996).

Recent development of the 'long PCR' technique, which employs two DNA polymerases (non-proofreading *Taq* and a proofreading *Pwo*) has led to the amplification of 10–40 kb fragments. This has opened up new possibilities for the rapid generation of RFLPs and mapping.

Amplification of full-length animal mitochondrial DNA by long PCR, by using a set of forward and reverse primers from the 16S RNA gene, has recently been reported (Nelson *et al.,* 1996). The size of mtDNA in animals ranges from 16 to 20 kb. However, amplification of full-length mtDNA from fungi is likely to be more challenging because the size of mtDNA in fungi varies between 20 and 170 kb (Taylor, 1986). Nonetheless, using appropriate primers, large fragments can be amplified by long PCR to generate RFLPs. Fragments of rDNA of up to 7 kb have been amplified from *Trichoderma* spp. with universal ribosomal primers (White *et al*., 1990) in long PCR, and used to detect RFLPs (S. Muthumeenakshi, Warwickshire, 1996, personal communication).

A rapid vector-independent restriction mapping technique has also been developed using long PCR. PCR primers are radioactively labelled to serve as probes for DNA fragments generated by partial digestion of the PCR product. This method has been used to generate restriction maps of 8–18 kb fragments directly amplified from human genomic DNA (Her and Weinshilboum, 1995).

Another application of long PCR is in repetitive-element based PCR (REP-PCR). Dispersed repeat sequences in *Magnaporthe grisea* are being used extensively to genotype the isolates by Southern hybridization-based fingerprinting (Levy *et al*., 1991; Xia *et al*., 1993). Recently, REP-PCR has

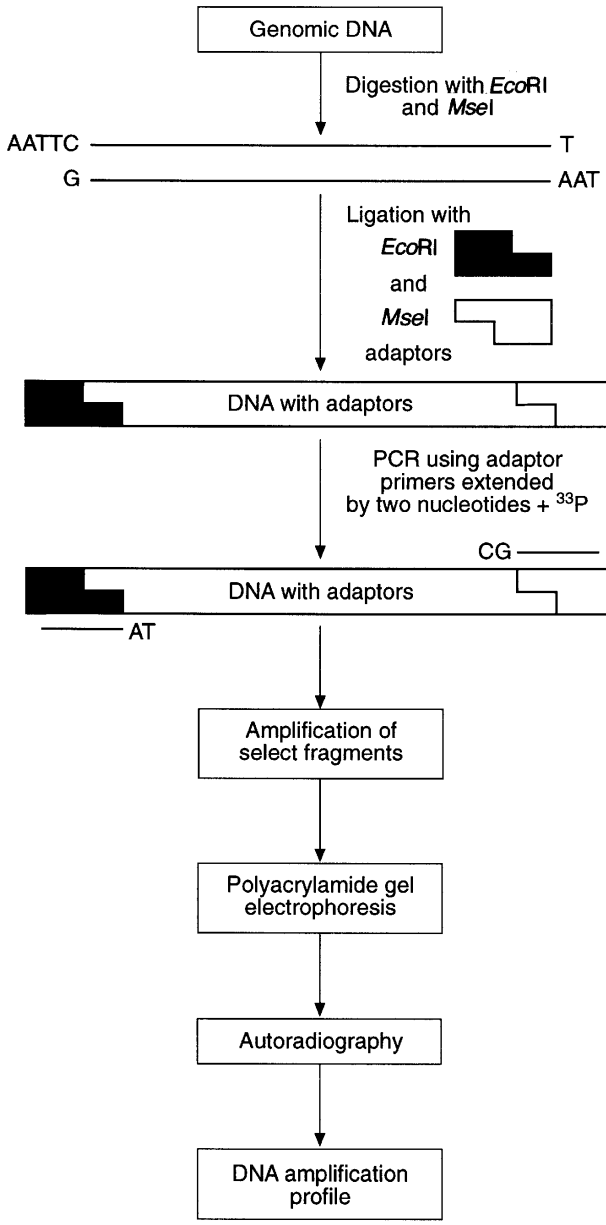

Fig. 15.1. Amplified fragment length polymorphism (AFLP) technique.

been developed for genotyping *M. grisea* isolates (George *et al*., 1997). Two outwardly directed primers, designed from a repetitive element sequence, were used in combination with long PCR to generate distinct banding patterns (Fig. 15.2) with *M. grisea* isolates from different hosts. The amplicons ranged in size from 0.4 kb to longer than 23 kb, although most scorable bands were less than 23 kb in size. The PCR profiles generated differentiated the rice-infecting and rice non-infecting *M. grisea* isolates. Robust groupings and close correspondence between the *Pot2* REP-PCR and MGR586 RFLP lineages were observed upon cluster analysis of PCR profiles from clonal as well as highly diverse rice-infecting *M. grisea* populations.

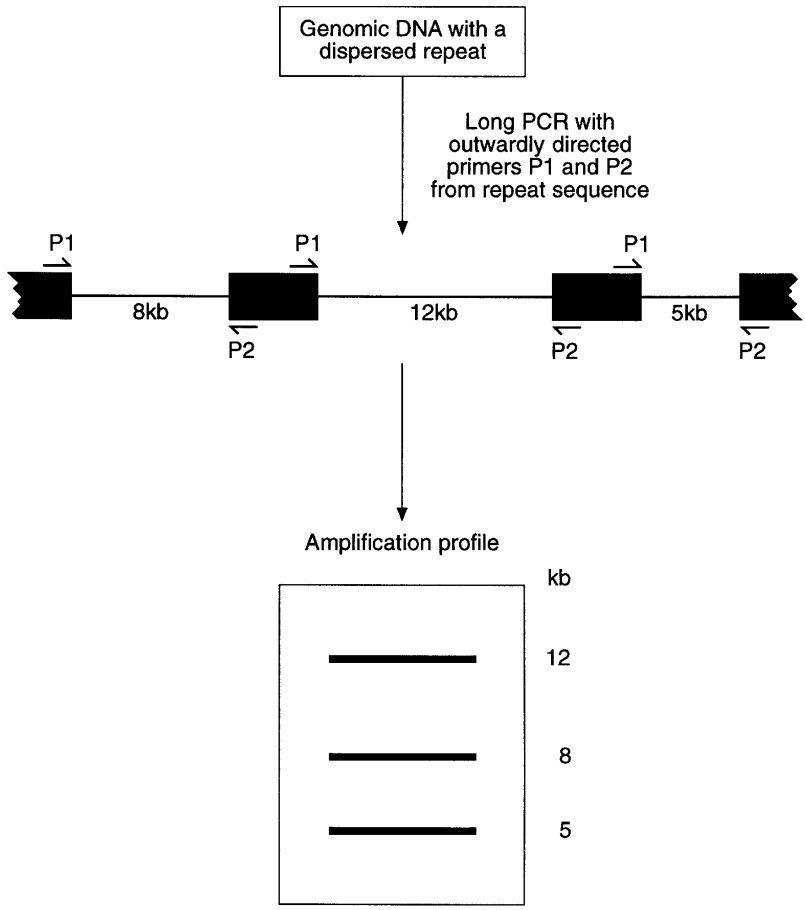

Fig. 15.2. Flowchart showing the REP-PCR technique.

15.3 Diagnostic PCR

PCR-based detection of pathogenic fungi directly from infected tissue has been reported for several important systems (Lévesque *et al*., 1994; Sreenivasaprasad *et al*., 1996). PCR has also been used to monitor mycorrhizal symbionts (Henrion *et al.,* 1994) and to detect pathogens directly from soil (Henson *et al*., 1993). Recently, a nested multiplex PCR approach has been developed to simultaneously detect conifer root rot fungi *Cylindrocarpon destructans* and *Cylindrocladium floridum* (Hamelin *et al*., 1996).

With most of these PCR methods, which are based on rDNA sequences, infected tissue with dead mycelium might also test positive as the DNA could still be amplified. It would be preferable to discriminate between viable and non-viable propagules of the pathogen, particularly in the context of certification programmes and quarantine tests. In order to detect the viable propagules only, it would be necessary to develop reverse transcriptase (RT)–PCR-based tests, using RNA as the source of amplification. Species-specific primers from rDNA sequences can be used in RT–PCR, although it is not certain whether amplification of ribosomal RNA can always be directly correlated with viable biomass. RT–PCR amplification of sequences such as chitin synthase genes and ergosterol biosynthetic genes can be more reliably related to the viability of the propagules. These RT–PCR tests based on gene sequences, however, are likely to be less sensitive as the abundance of these messages is much lower as compared with the ribosomal RNAs.

Currently, a number of diagnostic kits (Table 15.2) are available for routine use in forensic and clinical laboratories as a result of concerted research and development efforts between academia and industry, backed by strong funding. Similar advances can be envisaged for PCR application in mycology in biomedical fields.

Table 15.2. Some of the currently available forensic and clinical diagnostic kits (Graham, 1994; Whelen and Persing, 1996) as examples of the range of PCR applications.

Kit name	Basis of detection	Use
AmpliType PM	Five independently inherited genetic loci	Forensic testing and human identity
AmpliFLP D1S80	16 bp Repeat on chromosome one	Forensic testing and human identity
Enviroamp Legio.	5S Ribosomal RNA gene and macrophage *Legionella* infectivity potentiator gene	Detection of *Legionella*
Amplicor Myco.	16S Ribosomal RNA gene probe	Detection of *Mycobacterium tuberculosis*
Amplicor hep.	RT–PCR of viral RNA	Detection of hepatitis C virus

15.3.1 Data processing

The expected improvements in diagnostic capability necessitate the availability of software packages suitable for different applications. User-friendly interactive systems that answer questions in an efficient and simple manner are likely to facilitate the introduction of diagnostics into the user's environment.

15.4 Genome Mapping and Sequencing

PCR has contributed to rapid completion of physical maps of human and other genomes (Graham, 1994; Marx, 1995; Abramowitz, 1996). PCR was used as the basis for translating the various physical mapping markers into a common language of short fragments of single-copy DNA sequence called sequence-tagged sites (STSs). Generally, 200–500 bp of sequence data would define an STS. An STS is unique to that particular genome and can be specifically amplified by PCR. An STS detection PCR assay can be carried out using two primers (~ 20 bases) designed to be complementary to the two strands and at opposite ends of the sequence segment. Thus, a 100 kb clone can be mapped by determining the order of a series of STSs and measuring the distances between them. This information can be completely described in a database along with the conditions for the PCR assay (Olson *et al*., 1989). PCR has also had a considerable effect on genetic mapping through the amplification of the short tandem repeats (STRs) which are stretches of tandemly repeated short nucleotide motifs present in most eukaryotic genomes (Kashi *et al*., 1997). These elements occur as interspersed repeats and may be found by screening a genomic library. PCR primers can be designed for STR amplification by subcloning and sequencing (Litt, 1994). These STRs can be used both as polymorphic markers for measuring the frequency of recombination within families and also to interrelate physical and genetic maps. Further, substantial progress has been made in the analysis of complementary DNA clones to generate a segment of sequence called an expressed sequence tag (EST) by PCR as part of the Human Genome Project (White, 1996). A large volume of EST sequences is available from various animal and plant systems e.g. *Arabidopsis* (Newman *et al*., 1994). ESTs are generated by partial sequencing on cDNA clones. Clones from randomly primed cDNA libraries or partial cDNA clones can be used to generate this information rapidly. ESTs are useful for the identification and isolation of new genes, the identification of coding regions in genomic sequences and for genome mapping. PCR methods have also been used to map ESTs to specific chromosomes (Adams *et al*., 1991).

The wider application and success of genome sequencing projects depend on the availability of rapid and cost-effective sequencing technology. As part of the *Arabidopsis* genome sequencing initiative, a microthermal

cycler has been developed with which 0.5 µl sample volumes and 40 s cycle times can be achieved (Marziali *et al*., 1997). The two strategies presently employed are shotgun sequencing and primer walking. However, both of these approaches have time-consuming procedures. Large scale template preparation, generation of redundant sequences and the assembly of contigs are some of the major disadvantages with the former. With the latter, primer synthesis can be expensive and slow and automation is more complicated. The use of presynthesized libraries of primers with different sequences has been proposed to eliminate the primer synthesis step (Studier, 1989). Individual short primers from such a library are either ligated (Szybalski, 1990) or assembled without ligation (Beskin *et al*., 1995) to make unique sequencing primers. More recently, a conceptually new technique known as differential extension with nucleotide subsets (DENS) has been developed (Raja *et al*., 1997).

15.4.1 Differential extension with nucleotide subsets (DENS)

DNA sequencing using DENS is a two stage process (Fig. 15.3). Initially, a short primer is allowed to extend at 20–30°C with only two out of the four dNTPs present in the reaction mix. Primer (a partially degenerate octamer or a hexamer) extension depends on how soon a base corresponding to the dNTPs not used in the reaction is present in the strand being synthesized. This leads to differential extension of the primer, at different sites, depending on the template sequence. The intended priming site, the primer and the two dNTP subsets are chosen to maximize the length of the primer at that site. Elsewhere in the template, at randomly located priming sites, the primer extensions are likely to be substantially shorter. Subsequently, a termination reaction is performed at higher temperature with all four dNTPs present, similar to normal cycle sequencing. The higher annealing and extension temperature permits a sequence pattern to be generated only from the maximally extended site. Differential extension products of the same primer are shorter to anneal and extend and do not produce the sequence pattern. Although, at present, a <10% failure rate of DENS is expected because of the superimposed non-target sequence, further optimization of the procedures and conditions is likely to improve the usefulness of this technique.

With the increasing interest in mapping and sequencing of fungal genomes, particularly *Aspergillus* and *Neurospora* (Bennett, 1997; Hamer, 1997), it is likely that PCR will have a major impact in this area.

15.5 Gene Expression

Various life processes, such as development, differentiation and response to stress, are controlled by regulation of gene expression. Identification and

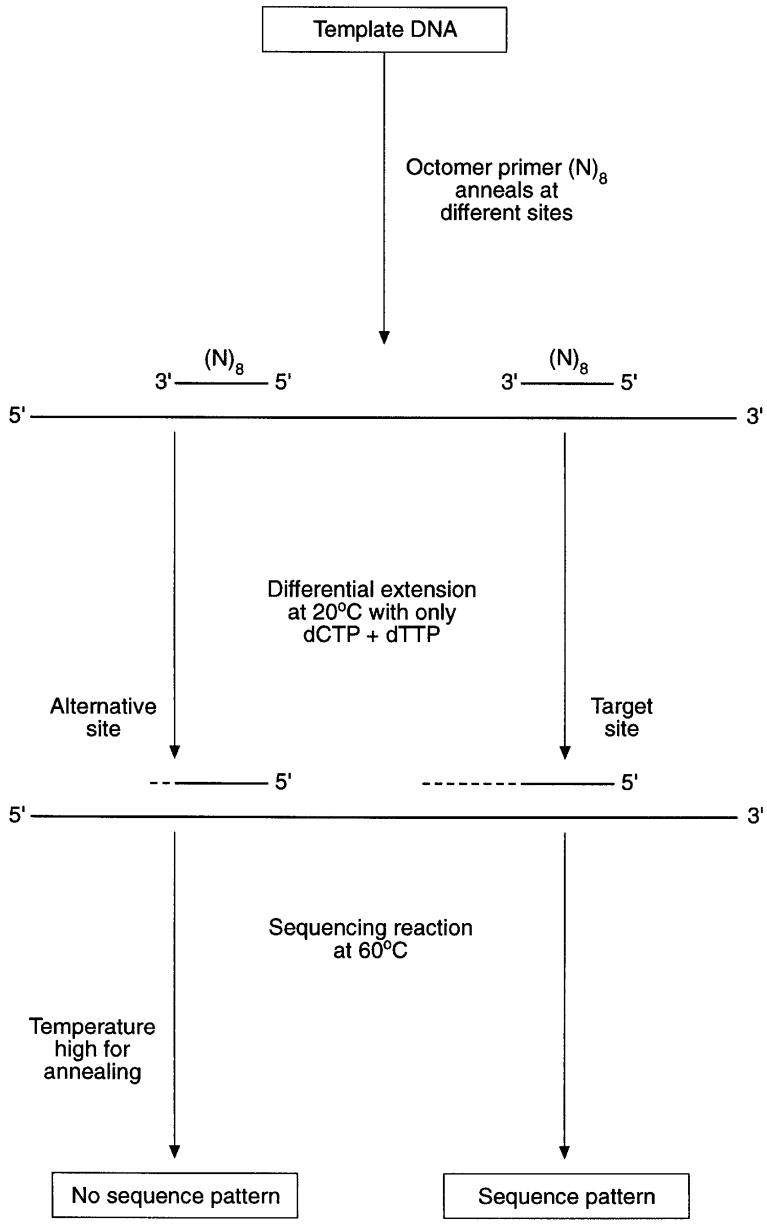

Fig. 15.3. Differential extension with nucleotide subsets technique for sequencing.

isolation of the differentially expressed genes are essential to understand and manipulate these biological processes. Conventionally, differential hybridization of cDNA libraries has been used to isolate these genes. Recently, various techniques that combine the advantages of PCR have been developed.

15.5.1 Differential display reverse transcription–PCR

Differential display reverse transcription–PCR (DDRT–PCR) was developed for isolating tumorigenesis-related genes from mice (Zhang and Medina, 1993) and humans (Liang *et al*., 1992), and has also been applied in the analysis of tomato fruit ripening-related genes (Oh *et al.,* 1995). The technique facilitates rapid isolation of differentially expressed genes via PCR without the construction and screening of cDNA libraries.

The total mRNA pool from a sample is reverse-transcribed into 12 subpopulations of cDNA using 12 different anchored oligo-dT primers (e.g. $T_{11}AC$). The cDNA subpopulations are then PCR-amplified using the same anchored primer and an arbitrary 10-mer. Additional cDNAs comprising each subpopulation are amplified by using alternative arbitrary primers (Fig. 15.4). The composite PCR profile is representative of the mRNAs contained in each population.

Radiolabelled amplification products derived from the same anchor/arbitrary primer combinations with different samples are displayed in adjacent lanes on polyacrylamide gels, to locate the differentially expressed genes. The gene fragments are excised, eluted, cloned and sequenced to identify the gene. These fragments can also be used as probes on genomic/cDNA libraries to clone complete genes.

DDRT–PCR is potentially faster than differential hybridization of cDNA libraries and initially gained wide attention. Despite this, some doubts have been raised as to the wider applicability of this technique as it might be biased for high copy number mRNA species and inappropriate where only a few genes might vary. A number of modifications have been made to the original protocol to optimize the technique, for example the use of longer primers to reduce the level of false positives, and to improve reproducibility. Several ready-to-use kits are now commercially available (e.g. Gene Hunter, Pharmacia).

15.5.2 Suppression PCR

This is a technique used to selectively amplify differentially expressed (target) cDNA fragments whilst simultaneously suppressing non-target amplification. Suppression PCR is specifically designed to equalize the abundance of messages within target cDNA to enable the isolation of even rare transcripts (Diatchenko *et al*., 1996).

Tester and driver double-stranded cDNAs are prepared from the two mRNA samples under comparison. Two tester populations are created with

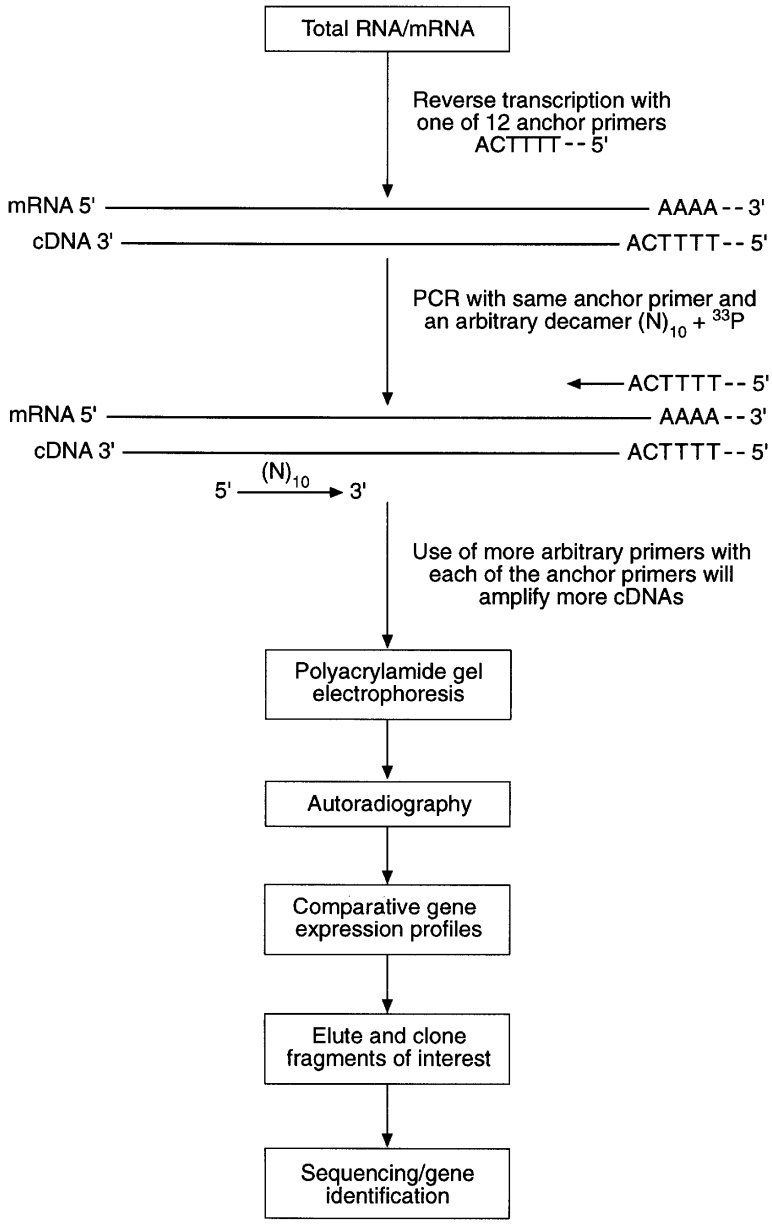

Fig. 15.4. RNA differential display technique.

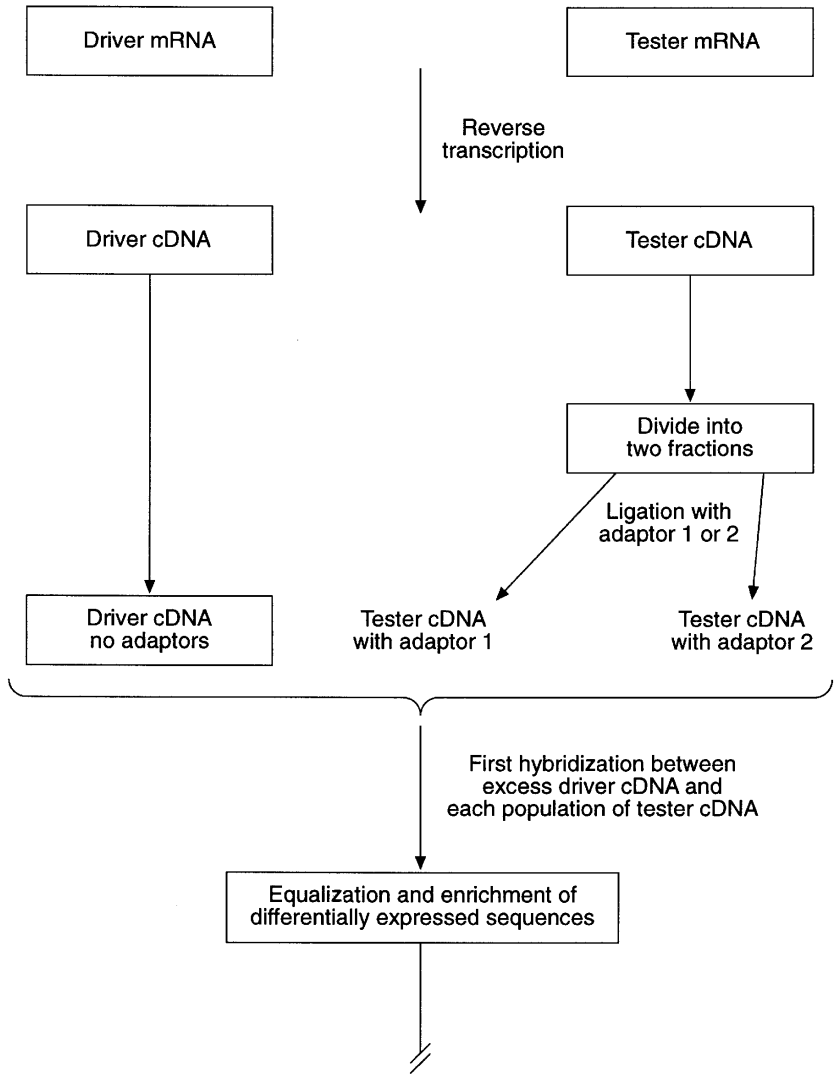

Fig. 15.5. Suppression PCR technique.

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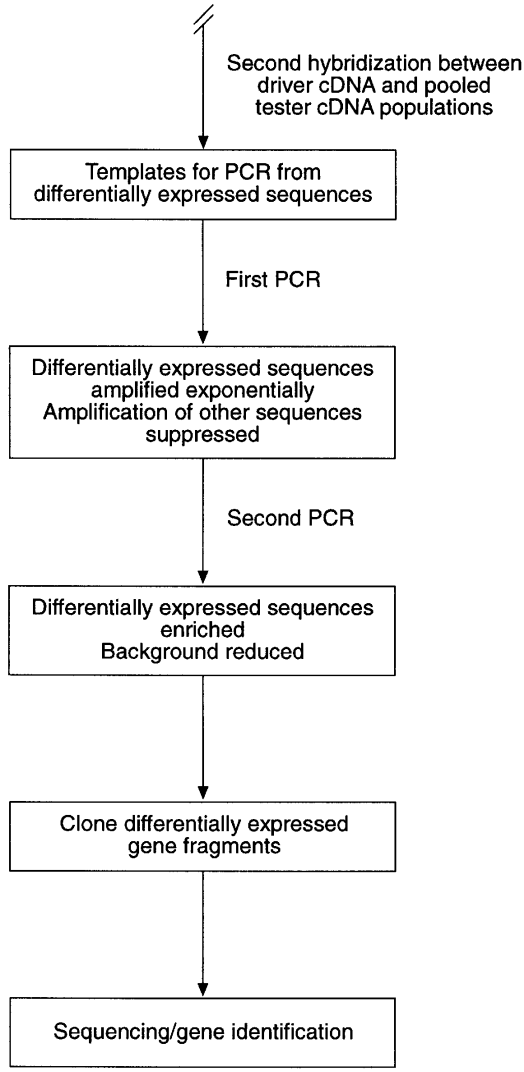

Fig. 15.5 continued.

different adaptors but the driver has no adaptors. The first hybridization between excess driver cDNA and each population of the tester cDNA results in equalization of message abundance and enrichment of differentially expressed sequences in the tester. The second hybridization between fresh driver cDNA and the two primary hybridization samples mixed together leads to the generation of differentially expressed gene templates for PCR.

Using suppression PCR, only the differentially expressed sequences are amplified exponentially during a first round PCR. A second round PCR with nested primers results in reduced background and enrichment of target sequences (Fig. 15.5).

This technique requires only 0.5–2.0 μg of poly(A)$^+$ RNA as starting material and is designed to amplify rare transcripts, typically the most difficult to obtain. Suppression PCR does not involve physical separation of single-stranded and double-stranded cDNA. Strategic design and use of adaptors suppress the amplification of most types of molecules including the molecules where panhandle-like structures are formed by the long inverted repeats. Suppression PCR can thus achieve greater than 1000-fold enrichment for differentially expressed cDNA (Clontech, 1995). A ready-to-use commercial kit is available from Clontech.

15.5.3 In situ *PCR*

This technique is used to amplify target DNA or RNA within intact cells, permitting the correlation of PCR results with cellular localization and morphology. Fixed cells or tissue are permeabilized and contained and PCR reagents are added to this. Thermal cycling through suitable parameters is followed by detection of PCR products using appropriate methods e.g. fluorescence microscopy. Lack of uniform equipment is perceived as the major problem in performing and reproducing *in situ* PCR. Techniques adapted for *in situ* thermal cycling, in particular, vary widely. With the availability of new *in situ* PCR systems (e.g. Perkin Elmer/Applied Biosystems) it is expected that this technique can be performed consistently, reproducibly and reliably (Perkin Elmer, 1994).

In situ PCR is an emerging tool in virology, cancer research and developmental biology. This technique is likely to be employed with various fungal systems. For example it has been applied in the study of arbuscular mycorrhizal (AM) fungi to confirm the presence of different alleles in AM fungal genes and to identify different nuclear populations within a single AM spore (Berta *et al*., 1996).

In the last few years, there has been an upsurge in understanding the molecular basis of differentiation and development in both pathogenic and beneficial fungi. Various PCR-based techniques are likely to be applied widely on a routine basis.

15.6 Amplification Technology

Rapid advances are being made in the miniaturization of the PCR instruments and also in reducing the time required for amplification. For example, by adapting PCR on to a microinstrumentation platform and using suitable detection chemistries in minute disposable chambers (0.5–50 μl), 30 cycles

can be completed in 10–20 min on a very small instrument (White, 1996). Similarly, the use of silicon chambers which require only 10 µl reactions and are heated resistively can reduce amplification time by an order of magnitude. Under these conditions 30 cycles may be completed in 7 min on a microfabricated prototype. Results for a variety of loci using these systems were comparable with the results from conventional PCR. This apparatus was about the size of a scientific calculator including power supply and control electronics and four 9 V batteries allowed 2.5 h of operation (Abramowitz, 1996). Such portable and convenient devices are likely to be used for on-field/on-site diagnosis.

Progress in the automation of nucleic acid extraction in combination with the advances in PCR technology are likely to lead to the availability of completely automated instrument systems for diagnostic clinics in the foreseeable future.

15.7 Detection Technology

A number of techniques (Graham, 1994) can be used to analyse PCR products (Table 15.3). Among these methods, scintillation proximity assay (Amersham), electrochemiluminescence (Perkin Elmer) and microwell assays (e.g. Roche) are suitable for analysing a large number of samples routinely.

Further progress is being made in developing novel amplicon analysis and detection methods. DNA can be bound to a 96-well polystyrene microtitre plate by non-covalent interactions e.g. by 500 mM NaCl or streptavidin coating, or immobilized probes can be used to capture biotinylated PCR products. The plate-bound amplicons can then be analysed by enzyme-assisted colorimetric detection which enables the simultaneous handling of numerous samples. A variation of this method is the use of a biotinylated primer and a modified triphosphate (digoxigenin-11-dUTP) in PCR (Fig. 15.6). Detection is achieved by capturing the biotinylated

Table 15.3. Amplicon analysis methods.

Electrophoresis
Hybridization
Direct sequencing
Laser/ALF detection
HPLC
Electrochemiluminescence
Scintillation proximity assay
Microwell assay

amplicons and using anti-digoxigenin antibody conjugated to alkaline phosphatase and suitable substrates (O'Donnell-Maloney *et al.*, 1996). Furthermore, by marking a specific primer with a unique hapten, e.g. digoxigenin or fluorescein, each reaction can be detected using hapten-specific antibodies labelled with different enzyme reporters, such as alkaline phosphatase or horseradish peroxidase. This system permits the detection of two different targets from a single reaction by different colour reactions (Tobe *et al.*, 1996).

Another method of amplicon detection is the use of a biotinylated primer and a fluorescently labelled primer in PCR. Streptavidin captured amplicons are denatured, the fluorescently labelled strand is hybridized to an array of immobilized probes on a glass slide and the hybridization pattern and polymorphisms are detected by fluorescence scanning (Fig. 15.7). An example of the use of this method is the identification of polymorphisms from exon 4 of the human tyrosinase gene (O'Donnell-Maloney *et al.*, 1996).

Detection methods that utilize reporter dyes are being integrated into new 'kinetic' thermal cyclers which can monitor the products during amplification. It is expected that these combined instrument–reagent systems

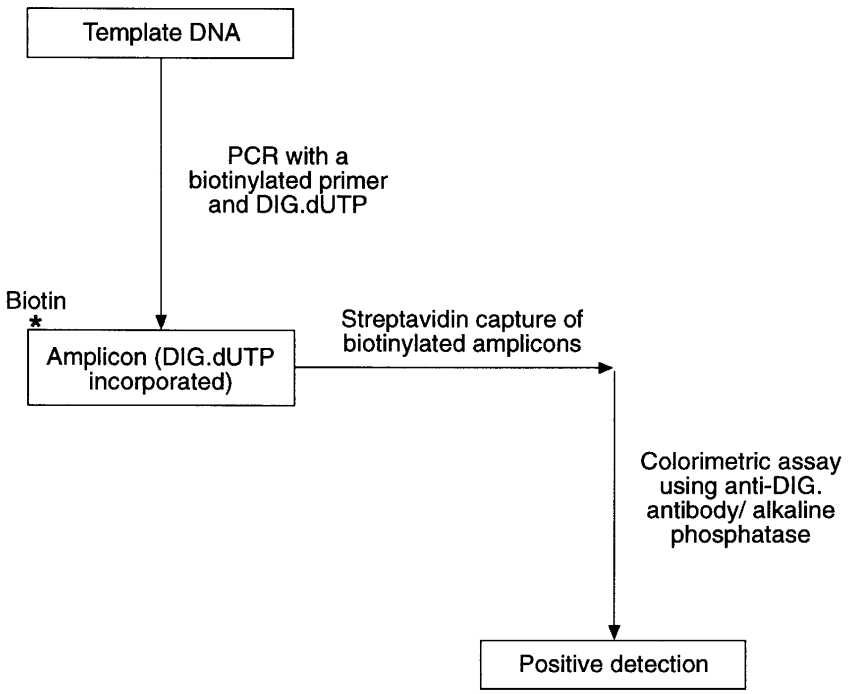

Fig. 15.6. Detection method using a colorimetric assay.

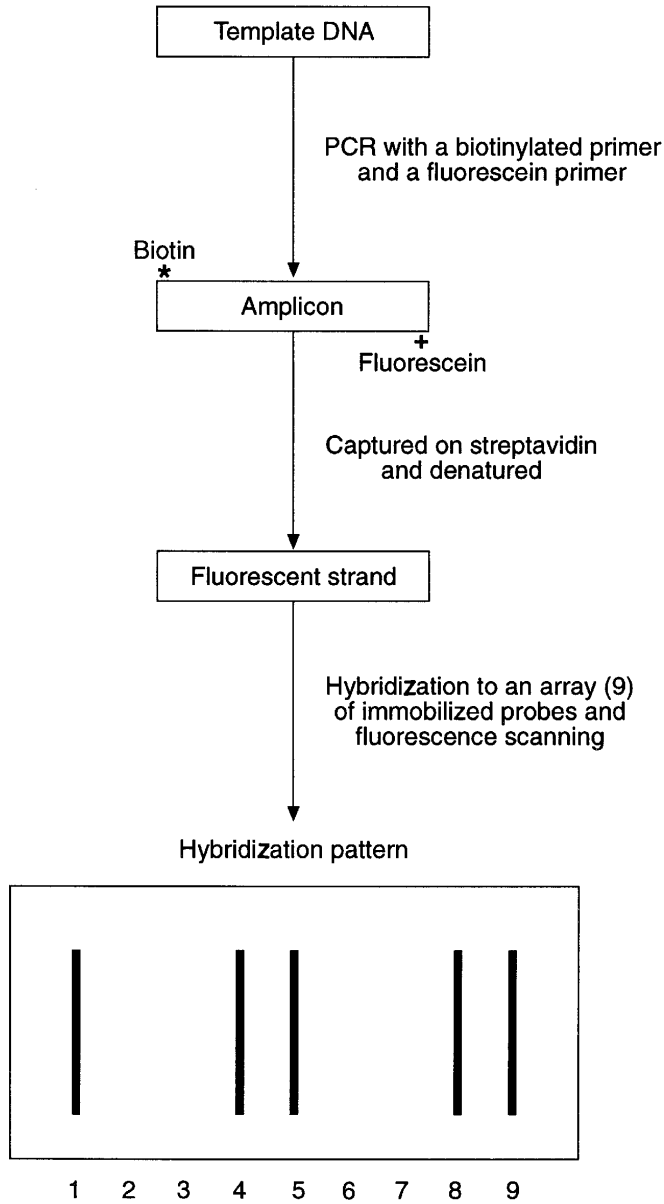

Fig. 15.7. Detection method based on amplicon hybridization to probe assays.

will enable rapid generation of both qualitative and quantitative data with high throughput (White, 1996). Recently, a microvolume multisample fluorimeter with rapid thermal cycling has been developed by Idaho

Technology, Idaho, USA. This instrument combines rapid thermal cycling capability, and fluorescent monitoring by three different techniques (Wittwer *et al*., 1997a, b).

Recently, scientists at USDA (Madison, Wisconsin) and Perkin Elmer (California) have applied an automated TaqMan assay system to detect *Tilletia indica* (Berthier-Schaad *et al*., 1997). The TaqMan system relies on the ability of the 5′ nuclease activity of *Taq* polymerase. During amplification, the reporter dye from the 5′ end of the probe is released and the resultant increase in fluorescence is monitored in real time. Under optimal PCR conditions, increase in fluorescent signal above the threshold level, by the end of the amplification cycles, detects a sample as positive for *T. indica.* Preliminary results on the comparison of classical PCR and TaqMan with *T. indica* from ryegrass and other hosts yielded approximately 60% agreement. With improvements in reliability, routine use of similar assay systems can be envisaged.

15.8 Conclusion

Rapid advances are being made in both amplification and detection technologies to enable rapid and high throughput PCR analyses, and the number of techniques available is continually increasing. In view of these developments, PCR is likely to be one of the most widely used tools in mycology in the broad context of genes and genomics as well as diagnostics.

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