POLYMERASE CHAIN REACTION (PCR) IN ARBUSCULAR MYCORRHIZA (AM) RESEARCH

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Introduction

DNA Polymerase is a naturally occurring enzyme, a biological macromolecule that catalyzes the formation and repair of DNA. It works by binding to a single DNA strand and synthesizing a complimentary strand. The accurate replication of all living matter depends on this activity, where it functions to duplicate DNA when cells divide.

PCR is a technique used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by *in vitro* enzymatic replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified. With PCR it is possible to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions of copies of the DNA piece.

The technique was first developed and used by Kary Mullis (Mullis, 1990) in 1983 for which he received the Nobel Prize a decade later. He successfully amplified a 110 base pair DNA fragment of the human b-globin gene using the *Eschericea coli* DNA polymerase and synthetic oligonucleotides sequencing unique genes to diagnose common human disease mutations. PCR has subsequently developed into a powerful tool used in all branches of molecular biology. In AM fungal research the technique has considerably clarified the phylogeny of Glomeromycota (Helgason *et al., 1998*; Redecker, 2002). The technique is now sensitive to the extent that DNA from a single AM fungal spore can be clearly identified (Gadkar

and Rillig, 2006; Settings, 2005; Schüßler *et al., 2001*), a new species classified (Chelius and Triplett, 1999) and a mix of AM propagules in soil (Rani *et al.,* 2004; Harrier, 2001) or colonization in roots (Helgason *et al.,* 1998; Redecker, 2000; Redecker *et al., 2003*) qualified.

An oligonucleotide is a defined non-biological short sequence of nucleic acids chemically synthesized from single stranded DNA molecules, usually 15-20 base pairs in length. In PCR, the oligonucleotide is referred to as a primer, allowing DNA polymerase to extend the oligonucleotide and replicate the complimentary strand. The primer is designed to mimic as closely as possible the targeted unique DNA strand of the organism being investigated.

The Procedure

In simplified terms, the procedure entails:

1. The extraction of DNA from the sample and denaturation of the helix into single strands by heating to >92°C for 20-30 seconds,
2. Annealing of the specifically designed primers to the ends of the required length of the single strand at lower temperatures, typically 50-65°C for 20-40 seconds,

3. Elongation or amplification of those specific lengths by the addition of the polymerase, the temperature raised to within a few degrees of the optimum activity temperature of the polymerase.

Taq, a commonly added heat resistant polymerase, is optimal at 75-80°C and usually a temperature of 72°C is used.

The cycle is repeated a further 19-39 times. There may be an initialization step prior to the first cycle and a final elongation after the last cycle at a temperature of 70-74°C for 5-15 minutes to ensure any remaining single-stranded DNA is fully extended.

Agarose gel electrophoresis is employed for size separation of the PCR products, determined by comparison with a DNA ladder run on the gel alongside which contains DNA fragments of known size.

Much of the procedure is now automated, e.g. DNA extraction, micro-array technology, high-throughput PCR cycle instrumentation (thermal cyclers), sequence analysers (Crow *et al.,* 2006; http://www.bio-rad.com, http://www.laboratorytalk.com/news/) or, enhanced by sophisticated software as in primer design (Alkan *et al., 2006*; Estruch, 2008; http://www.idtdna.com/). Gene base pair and primer sequences are readily available in the public domain for easy reference (http://www.invam.caf.wvu.edu/; http://www.ncbi.nlm.nih.gov/ GenBank and/Basic Local Alignment Research Tool, http://www.kent.ac.uk/bio/beg/; http://www.bio.ukc.ac.uk/IIBMIRCEN/, http://www.ncbi.nlm.nih.gov/;http://www.ebi.ac.uk/embl European Molecular Biology Laboratory (EMBL)).

[**NOTE:** There are many errors in the databases, particularly contaminants (Schüßler *et al., 2003*; http://www.amf-phylogeny.com)].

The search for more efficient heat stable polymerases is under way (http://www.freepatentsonline.com/6673585.html & /EP0547359.html).

Hot Start activation strategies are being developed (Lebedev *et al.,* 2008), as are nested-PCR techniques (van Tuinen *et al.,* 2002; Reddy *et al.,* 2005), reverse transcription PCR (RT–PCR) (Xu *et al., 2008*), intersequence-specific PCR (ISSR) (Pawlowska, 2005) and quantitative PCR (Redecker, 2000, Alkan *et al.,* 2004).

Still, however, there remain variables which must be addressed on each occasion: the length of the target DNA template;

reaction volumes of extract, polymerase, buffer components, manganese or magnesium divalent cations, monovalent potassium ions and deoxynucleoside triphosphates; temperatures of each of the three steps i.e. denaturation, annealing and elongation, in the thermal cycles and the amount of time applied; the number of cycles and, perhaps the most critical of all, primer specificity.

Some Problems in PCR

Each and every step of the procedure and allocation of the variables listed above may give rise to discrepancies.

Contamination of the DNA extracted from the sample is probably the most common cause. The contaminants may include bacterial DNA from a spore surface, a mycobiont within the spore, host plant DNA and even human DNA from handling. As new approaches to specificity develop, there is increment in the exclusion of the amplification of contaminant DNA, but care should be taken to follow prescribed laboratory protocols (http://www.clarku.edu.faculty/dhibbett/protocols folder/Lab protocols/.pdf, http://www.wheat.pw.usda.gov/~lazlo/methods/).

Cycle temperatures affect the outcome. The most critical are those for denaturation and annealing. Denaturation temperatures too low (<90°C) result in lower yields or non-specific products: too high they may damage the enzyme reducing, even eliminating, yield.

Annealing temperature too low results in non-specific amplification: too high, then low or even no yield.

Small fragments of DNA are usually easily amplified but longer templates will exponentially amplify any inherent error in the DNA. Typically 0.1 – 5 kilo base pair (kb) size range sequences are used, often at the lower end.

Taq polymerase is the enzyme most commonly used in PCR, a heat resistant polymerase derived from the thermophilic bacterium *Thermus aquaticus* (Taq), its purification and commercial distribution contributing considerably to automation of the procedure. However, because it has no 3' to 5' error-proofreading activity, error rate is high, approximately 1 in 6,000 bases. If misincorporations occur during the first few cycles of amplification, in a 1 kb sequence that has undergone 20 effective cycles of duplication approximately 40% of the new DNA strands synthesized may contain an incorrect nucleotide resulting from a copying error, as the effect tends to be cumulative (van Pelt-Verkuil *et al., 2008*).

Non-specific binding of primers frequently occurs and can be due to repeat sequences in the DNA template, non-specific binding between primer and template, and incomplete primer binding, leaving the 5' end of the primer unattached to the template. Non-specific binding is also often increased when degenerate primers are used in the PCR.

Primer Design
10 tips for primer design:

1. Design your PCR primers to be 18-30 oligonucleotides in length. The longer end of this range allows higher specificity and gives you space to add restriction enzyme sites to the primer end for cloning.

2. Make sure the melting temperatures (Tm) of the primers used are not more than 5°C different from each other. You can calculate Tm with this formula: Tm = 4(G + C) + 2(A + T)°C.

3. Aim for a Tm between 65 and 70°C for each primer over the region of hybridization.

4. Use an annealing temperature (Ta) of 10 to 15°C lower than the Tm.

5. The GC content of each primer should be in the range of 40-60% for optimum PCR efficiency.

6. Try to have uniform distribution of G and C nucleotides, as clusters of G's or C's can cause non-specific priming.

7. Avoid long runs of the same nucleotide.

8. Check that primers are not self-complementary or complementary to the other primer in the reaction mixture as this will encourage formation of hairpins and primer-dimers and will compete with the template for the use of primer and reagent.

9. If you can, make the 3′ end terminate in C or A, as the 3′ is the end which extends and neither the C nor A nucleotide wobbles. This will increase the specificity.

10. You can help avoid mis-priming by making the 3′ end slightly AT rich.

PCR/Traditional Morphological Techniques

There is no doubt PCR has revolutionized, just as in all other applications of the technique, research in AM fungi. Where morphological techniques may be applied to identify AM fungi from spore description and, to some extent hyphal characteristics, to the species level, the phylogeny of Glomeromycota, the detection of species which are neither sporulating nor staining, the number and identification of different species colonizing the root and the description of propagules in soil, may be assessed by PCR. PCR is rapidly becoming an essential laboratory technique in the field of mycorrhizal research.

References

Alkan, N., V. Gadkar, J. Coburn, O. Yarden, Y. Kapulnik (2004). Quantification of the arbuscular mycorrhizal fungus *Glomus intraradices* in host tissue using real-time polymerase chain reaction. *New Phytol.* 161, 877-885.

Alkan, N., V. Gadkar, O. Yarden, Y. Kapulnik (2006). Analysis of quantitative interactions between two species of arbuscular mycorrhizal fungi, *Glomus mosseae* and *G. intraradices,* by Real-Time PCR. *Appl. Environ. Microbiol.* 72, 4192-4199.

Chelius, M.K., E.W. Triplett (1999). Rapid detection of arbuscular mycorrhizae in roots and soil of an intensively managed turfgrass system by PCR amplification of small subunit rDNA. *Mycorrhiza* 9, 61-64.

Crow, R.M., J.S. Gartland, A.T. McHugh, K.M.A. Gartland (2006). Real-time GUS analysis using Q-PCR instrumentation. *J. Biotech.* 126, 135-139.

Estruch, J.J. (2008). OLIGO primer selection. In: Methods in Microbiology. Sequence Data Analysis Guidebook 70, 279-285. (Ed. S. R. Swindell). Humana Press: ISSN online 1940-6029.

Gadkar, V., M.C. Rillig (2006). Application of Phi29 DNA polymerase mediated whole genome amplification on single spores of arbuscular mycorrhizal (AM) fungi. *FEMS Microbiol. Letters* Vol. 242, 65-71.

Harrier, L.A. (2001). The arbuscular mycorrhizal symbiosis: A molecular review of the fungal dimension. *J. Exp. Bot.* 52, 469-478.

Helgason, T., T.J. Daniell, R. Husband, A.H. Fitter, J.P.W. Young (1998). Ploughing up the wood-wide web? *Nature* 394, 431.

http://www.amf-phylogeny.com List with comments about mislabeled sequences and tables of contaminant sequences.

http://www.bio.ukc.ac.uk/IIBMIRCEN/ La Banque Europeenne des Glomales (BEG) INRA, Dijon Cedex, France.

http://www.bio-rad.com

http://www.clarku.edu/faculty/dhibbett/protocols_folder/Lab_protocols/.pdf Hibbett lab protocols for DNA isolation, PCR, and DNA sequencing.

http://www.ebi.ac.uk/ embl/ European Molecular Biology Laboratory (EMBL).

http://www.freepatentsonline.com/6673585.html Heat-stable DNA Polymerase of *Archaeobacteria* of genus *Pyrococcus* sp.

http://www.freepatentsonline.com/EP0547359.html Purified thermostable DNA Polymerase obtainable from *Pyrococcus* species.

http://www.idtdna.com/ Integrated DNA Technologies Inc., USA.

http://www.invam.caf.wvu.edu/ International Collection of Arbuscular and Vesicular Arbuscular Mycorrhizal Fungi, Morgantown, USA.

http://www.kent.ac.uk/bio/beg/ International Bank for the Glomeromycota.

http://www.laboratorytalk.com/news/

http://www.ncbi.nlm.nih.gov/ Basic Local Alignment Search Tool (BLAST).

http://www.ncbi.nlm.nih.gov/ GenBank.

http://www.wheat.pw.usda.gov/~lazlo/methods/.

Lebedev, A.V., N. Paul, J. Yee, V.A. Timoshchuk, J. Shum, K. Miyagi, J. Kellum, R.I. Hogrefe, G. Zon (2008). Hot start PCR with heat-activatable primers: A novel approach for improved PCR performance. *Nuc. Acids Res.* 36, 131.

Mullis, K.B. (1990). The unusual origin of the Polymerase Chain Reaction. *Sci. Am.* 262, 56-61.

Pawlowska, T.E. (2005). Genetic processes in arbuscular mycorrhizal fungi. *FEMS Microbiol. Lett.* 251, 185-192.

Rani, S.S., I.K. Kunwar, G.S. Prasad, C. Manoharachary (2004). *Glomus hyderabadensis*, a new species: its taxonomy and phylogenetic comparison with related species. *Mycotaxon* 89, 245-253.

Reddy, S.R., P.K. Pindi, S.M. Reddy (2005). Molecular methods for research on arbuscular mycorrhizal fungi in India: Problems and prospects. *Curr. Sci.* 89, 1699-1709.

Redecker, D. (2000). Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. *Mycorrhiza* 10, 73-80.

Redecker, D. (2002). Molecular identification and phylogeny of arbuscular mycorrhizal fungi. *Plant and Soil* 224, 67-73.

Redecker, D., I. Hijri, A. Wiemken (2003). Molecular identification of arbuscular mycorrhizal fungi in roots: Perspectives and Problems. *Folia Geobot.* 38, 113-124.

Schüßler, A., D. Schwarzott, C. Walker (2003). Glomeramycota rRNA genes – the diversity of myths? *Mycorrhiza* 13, 233-236.

Schüßler, A., H. Gehrig, D. Scharzott, C. Walker (2001). Analysis of partial *Glomales* SSU rRNA gene sequences: implications for primer design and phylogeny. *Mycol. Res.* 105, 5-15.

Settings, M. (2005). Genetic markers in single spores of AM fungi. *Mycol. Res.* 109, 130-131.

van Pelt-Verkuil, E., A. van Belkum, J.P. Hays (2008). Principles and technical aspects of PCR amplification. Springer. *pp.* 107-117.

van Tuinen, D., E. Jacquot, B. Zhao, A. Gollotte, V. Gianinazzi-Pearson (2002). Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. *J. Mol. Ecol.* 7, 879-887.

Xu, P., P. Christie, Y. Liu, J. Zhang, X. Li (2008). The arbuscular mycorrhizal fungus *Glomus mosseae* can enhance arsenic tolerance in *Medicago truncatula* by increasing plant phosphorus status and restricting arsenate uptake. *Environmental Pollution* 156, 215-220.

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