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From PCR to RCA: a surgical trainee's guide to the techniques of genetic amplification

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With the advent of evidence-based medicine and the Calman–Hine Report, more and more surgical trainees are undertaking a period of research, either before entering or during their Specialist Registrar training. Many will encounter concepts in science uncommon in daily clinical settings. This paper will elucidate the techniques of genetic amplification available today with their potential for usage in clinical research.

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INTRODUCTION

In this Calman era of postgraduate training, research in any of its forms and guises has become an integral part of a surgeon's training. More and more trainees are undertaking a period of a year, or two, of research prior to applying for a Specialist Registrar post within the National Health Service. As such, we as a profession have to get accustomed to terms and techniques that were once the sole domain of scientists. In the fields of molecular biology and diagnostic genetics, the concept and technique of the polymerase chain reaction has been central to most of its major recent advances and forms the catalyst for things to come. Prior to 1986, investigation into genetics was hampered by the lack of abundance of genetic material, be it a gene sequence or even a protein by-product of a particular reaction. The only way to obtain a large quantity of DNA was by performing Recombinant DNA cloning. This enormously laborious and lengthy task was in essence an *in vitro* replica of the mitotic process that occurred naturally; producing clones of the original material in its entirety, not targeting the specific area under investigation. The time needed to produce a significant amount of genetic material was usually in excess of 96 hours and involved a multitude of steps.[1] This was certainly not ideal. Then in 1983 the rules of genetic amplification changed forever.

Kary Mullis, a research scientist with the Cetus Corporation in California, USA required large quantities of DNA material for a project he was involved with. Unhappy with the then standard practice of recombinant DNA cloning, he devised an entirely new way of genetic amplification. According to Mullis, this happened while he was driving in Mendocino County late one evening, and I quote, 'We were at mile marker 46.58 on Highway 128, and we were on the very edge of the dawn of the age of PCR.'[2]

WHAT IS PCR?

At its heart, the concept behind the polymerase chain reaction is a basic one. It utilizes the natural cleaving ability of the polymerase enzyme within a repetitive multi-step protocol to form large copies of a targeted gene sequence. Two primers are used, each a short chain oligonucleotide, to bind to opposing ends of a specific genetic sequence. The polymerase enzyme then cleaves this specific area producing a single strand of DNA. By a process of rapidly heating and cooling the genetic material, under certain specific conditions, new double stranded DNA will be formed.[3,4] The number or products formed, from one copy of genetic material, after 10 cycles is 1024 but due to the exponential capabilities of this system, the number produced after just 20 cycles is in excess of a million copies. PCR has superceded recombinant DNA cloning because it offers the user two significant improvements on the old system;

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one, the ability to produce large quantities of DNA within a relatively short space of time and two, these reactions are self-propagating as long as all the essential ingredients are present along with the ideal conditions.

PCR SYSTEMS

There exist various types of PCR systems, but they can be divided into two broad categories, Standard and Quantitative. The starting point of the original PCR systems is genomic DNA harvested from total mRNA by a process of reverse-transcription. The DNA to be amplified is then added, along with the primers for a targeted genetic sequence, to a micro-centrifuge tube containing a pre-determined amount of DNA polymerase, dNTPs and salts.[5] Reverse transcription (RT) then occurs within this closed tube environment. The cDNA produced is then transferred to a thermal cycler where the actual mechanics of the PCR process occur, i.e. the rapid heating, to dissociate the DNA strands; cooling, to allow the strands and primers to anneal together and extension, to allow DNA synthesis. After a period of time, depending on the number of cycles performed, the products were then placed into a gel electrophoresis system to detect the products produced. Direct quantitation was not possible on the original systems.[6]

Quantitative PCR solves this problem, but does it in a variety of ways.[7] Real-time PCR is the latest available PCR system available.[8,9] Its role has expanded since the invention of fluorogenic primer-probe sequences. Here, a 20–30 base pair oligonucleotide called a probe is attached to the region under investigation. It lies entirely within the region described by the two primers. These probes have 2 completely separate but equally important ends. One end contains a fluorophobe, while attached to the other is a fluorescent-quencher. These two attachments lie in close proximity to each other while the probe remains intact. Once annealing occurs, they separtate. Thus, detection of products is made possible at the end of each cycle by 'released fluorescence'.[10,11] Quantitation is achieved by concurrent running of PCR on tubes containing serial dilutions of known copy numbers of the genetic material in question. A logarithmic graph can thus be plotted and any quantity can be determined based on the level of fluorescence as well as the time at which the amplification process actually began.[8]

OTHER FORMS OF GENETIC AMPLIFICATION

Whilst PCR remains the most common method of genetic amplification, it is by no means the only way to amplify DNA. Strand Displacement Amplification (SDA), Ligase Chain Reaction (LCR) and Transcription Mediated Amplification (TMA) are three variations on the basic PCR concept, while Invader Amplification (IA) and Rolling Circle Amplification (RCA) utilize completely different methods of DNA amplification, concentrating on the DNA signals produced rather than the specific DNA target sequence.

SDA varies from PCR in that it does not require the exonuclease activity of a DNA polymerase as well as not requiring any significant thermocycling. It comprises of two distinct, but complimentary steps, namely Target Generation and Exponential Target Amplification. Two primer sets are used to select a targeted area of dsDNA via a denaturing and hybridization process. A dual dye hairpin probe with its ubiquitous quenchers detects the targeted area and anneals to it. During the process of amplification, the quenchers are cleaved allowing fluorescence to be detected.[12]

LCR, as its name suggests, differs from PCR by utilizing the covalent binding properties of a DNA ligase, instead of the exonuclease activity of a DNA polymerase. The ligase binds two probes to opposing ends of a targeted region of DNA. The rest of the process is similar to PCR in that rapid thermocycling is required to amplify the products.[13]

TMA attempts to decrease the number of variables during genetic amplification by producing a closed-isothermal reaction environment, once all the various components are in place. RNA polymerase as well as reverse-transcriptase is present within this closed environment to allow amplification of either RNA or DNA strands. Products are detected by released fluorescence.[14]

The IA system uses two separate probes, which overlap on annealing to the appropriate target sequence. These probes differ greatly in their form and structure and only the 'downstream-probe' contains the fluorescent dye. A thermostable endonuclease is added to the mixture at elevated thermal levels, the products are cleaved. The 'upstream-probe' usually has a higher melting point than the one located downstream and therefore remains attached to the DNA target sequence, thus driving the process forward. Upon cleavage, the 'downstream-probe' releases its fluorescence, which can be detected on a standard fluorescence plate reader. This method is extremely specific as mispairing by even one base pair prevents the initial overlap of probes, which begins the cycle. In this way IA is extremely useful in investigating single point mutations in genes.[15]

RCA is potentially the most flexible and accommodating method of genetic amplification. It is a novel way of using circularised oligonucleotides to amplify either DNA signals or sequences. A circular probe with a 'gap region' attaches to a single stranded DNA target. The gap is then filled by either a DNA polymerase or a 5′ phosphorylated oligonucleotide. Once the gap is filled the target sequence is now safely bound to the circular probe. Ligation of this area prior to activation by a phage

DNA polymerase creates new circular structures; if however, the polymerase was allowed to act in the absence of a ligase, a linear single strand copy is formed. The two main attractions of this technique are its exquisite specificity and the potential of exploiting the fact that each circular probe is in essence a 'closed-loop' and attaching multiple probes, each with its own individual fluorescent dye, onto various sites on a single target gene. In this way multiple targets can be investigated concurrently under the same thermal conditions.[16]

As IA and RCA amplify DNA signals, they are subject to different problems than those associated with PCR. While with PCR based systems, exponential target amplification may lead to the possibility of amplicon cross-contamination, signal based systems have the problem of insufficient signal generation to allow direct analysis without actual coupling to a secondary amplification step to continue target amplification.

CONCLUSION

As more and more surgical trainees undertake periods of research during their SpR training, we will have to re-learn our scientific skills. The field of molecular biology provides a most exciting backdrop for surgical investigation. The ability to detect small genetic mismatches or even to detect cancer cells *in vivo* are made possible by genetic amplification. What was initially a laborious and tedious process lasting a period of a few days is now a simple process that provides amplification of more than 12 orders of magnitude, within the space of a few hours. Substantial new discoveries and refinement of available technology are responsible for pushing back the boundaries of what can be learnt from a single genetic sequence. May we, collectively, embrace and continue to add to these discoveries.

REFERENCES

1. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual 2nd Edition*. 1989. Cold Spring Harbor Laboratory Press.
2. Mullis KB. *Dancing Naked in the Mind Field*. 2000. Vintage Books.
3. Mullis KB, Ferre F, Gibbs RA (Eds). *The Polymerase Chain Reaction*. 1994. Boston: Birkhauser.
4. Mullis K, Faloona F, Scharf S, Sanki R, Horn G, Erlich H. Specific enzymatic amplification of DNA *in vitro*: The Polymerase Chain Reaction. *Cold Spring Harbor Symp. Quant. Biol* 1986; **51**: 263–73.
5. Hengen PN. Quantitative PCR: An accurate measure of mRNA. *Trends Biochem Sci* 1995; **20**: 476–7.
6. Halford WP, Falco VC, Gebhardt BM, Carr DJJ. The inherent quantitative capacity of the Reverse Transcription Polymerase Chain Reaction. *Anal Biochem* 1999; **266**: 181–91.
7. Raeymakers L. Quantitative PCR: Theoretical considerations with practical implications. *Anal Biochem* 1993; **214**: 582–5.
8. Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR: Real time monitoring of DNA amplification reactions. *Biotechnology* 1993; **11**: 1026–30.
9. Higuchi R, Dollinger G, Walsh PS, Griffith R. Simultaneous amplification and detection of specific DNA sequences. *Biotechnology* 1992; **10**: 413–17.
10. Lee LG, Connell CR, Bloch W. Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucleic Acids Res* 1993; **21**: 3761–6.
11. Livak KJ, Flood SJA, Marmero J, Giusti W, Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods and Applications* 1995; **4**: 357–62.
12. Little MC *et al*. Strand displacement amplification and homogenous real-time detection incorporated in a second-generation DNA probe system BDProbeTecET. *Clin Chem* 1999; **45**: 777–84.
13. Barany F. Genetic disease detection and DNA amplification using cloned thermostable ligase. *Proct Natl Acad Sci USA* 1991; **88**: 189–93.
14. Schweitzer B, Kingsmore S. Combining nucleic acid amplification and detection. *Current Opinion in Biotechnology* 2001; **12**: 21–7.
15. Hall J, Eis P, Law S, Reynaldo L, Prudent J, Marshall D, Allawi H, Mast A, Dahlberg J, Kwiatkowski R, de Arruda M, Neri P, Lyamichev V. Sensitive detection of DNA polymorphism by the serial invasive signal amplification reaction. *Proct Natl Acad Sci USA* 2000; **97**: 8272–7.
16. Lizardi P, Huang X, Zhu Z, Bray-Ward P, Thomas D, Ward D. Mutation detection and single molecule counting using isothermal rolling circle amplification. *Nat Genet* 1998; **19**: 225–32.

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