NORTHERN ILLINOIS UNIVERSITY

"PCR, Its Development & Application to My Research"

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by

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Introduction to the Polymerase Chain Reaction

In 1986 an article was published in the Cold Spring Harbor 51st. Symposium on Quantitative Molecular Biology which detailed the discovery of the polymerase chain reaction. Ironically, the original idea for this world changing discovery was developed in 1971 by Kleppe. However, Kleppe did not pursue his insight with further experiments and it was Mullis and his colleges who published the technique which could be used to amplify large amounts of copy DNA or cDNA, from a small amount of DNA (Mullis et. al., 1986). Every discovery must fit into history, and it is curious to note that at the same time PCR was being developed, the computer was making inroades into the biological sciences. Even in the Mullis paper, this new application of computers in science is observed by his primitive computer animated diagrams of PCR. The computer also made it possible to handle the explosion of DNA and protein sequence information that would have undoubtedly burdened countless graduate students attempting to plug and chug their way through countless pages of sequence information.

The polymerase chain reaction (PCR) is a procedure for the rapid in vitro enzymatic amplification of a specific segment of DNA(Kramer et. al,

1997). The reaction requires three different nucleic acids: a double stranded DNA template and two 10-30 base DNA primers that bind to the 5′ coding and 3′ noncoding DNA strand respectivly. There is also a DNA polymerase and the nucleic acid replication reagents such as ATP, dNTP's, buffers and salts. The original DNA, PCR primers, dNTP's, buffers and salts are combined in a tube. The DNA is denatured by heating the sample to 94° for 5 minutes and then allowed to cool to 65°. Since the primers are in excess they bind to their appropriate DNA sequence separating the two DNA strands. The polymerase then begins the process of DNA replication. The tube is then cooled and the first cycle of PCR is complete. The process is repeated cycle after cycle until billions of copies of the selected DNA gene have been created.

In this paper I will cover the history and development of the polymerase chain reaction, its applications and how I have used PCR in my research project.

History and Development of the Polymerase Chain Reaction.

In 1971 in the Journal of Molecular Biology K. Kleppe wrote "The synthesis of the duplexed tRNA genes which emerge from the present work are as follows; the DNA duplex would be denatured to form single strands.

This denaturation step would be carried out in the presence of a sufficiently large excess of the two appropriate primers. Upon cooling, one would hope to obtain two structures, each containing the full length of the template strand appropriately complexed with the primer. DNA polymerase will be added to complete the process of repair replication. The molecules of the original duplex should result. The whole cycle could be repeated, there being added every time a fresh dose of the enzyme."(Kleppe et. al., 1971) At the conclusion of the paper Kleppe indicated that research was being done along these lines, however, no paper was ever published. This was definitely the guy who missed the boat.

The development of PCR has created some interesting problems, the first being PCR has made DNA research boring. "Projects that formerly required some subtle deduction, clever manipulation, special insight, or good fortune are now within the reach of anyone willing to assemble a few reagents and a cycler, and follow a well-worn routine. (Mullis et. al., 1994)". The second problem is that PCR is painfully brilliant. Brilliant because of its remarkable simplicity and awesome effectiveness and painful because they didn't think of it themselves. As to this Mullis answers with an old Bob Dylan refrain, "Can I help it, if I'm lucky?" Or unlucky if your Kleppe. The third problem arises from the fiasco that resulted from the copyright on PCR by Cetus which in itself would be the topic of another long paper.

Kary Mullis is not just the inventor of PCR. In addition to the polymerase chain reaction he is also known for inventing plastic that changes color when exposed to ultraviolet light, as well as publishing a paper in nature intitled "The Cosmological Significance of time reversal." He describes himself as "a generalist with a chemical prejudice." Kary Mullis earned his masters and his Ph.D. in biochemistry, at the University of California, Berkeley. After completing his Ph.D. in 1972, he worked as a postdoctoral fellow at the university of Kansas Medical School and the University of California, San Francisco. Following his postdoctoral work, he joined the Cetus corporation, where he worked on the polymerase chain reaction. In 1986 he became the director of molecular biology at Xytronyx, Inc. and now works as a private consultant on the polymerase chain reaction and nucleic acid chemistry.

One Friday night in April 1983, while traveling with a colleague along a moonlight mountain road into northern California's redwood country, Mullis stumbled on a idea, the polymerase chain reaction, that would change molecular biology and eventually win him the Nobel Prize. Since that moonlight night, more than a thousand uses for PCR have been published and the applications of PCR have spread throughout biological sciences.

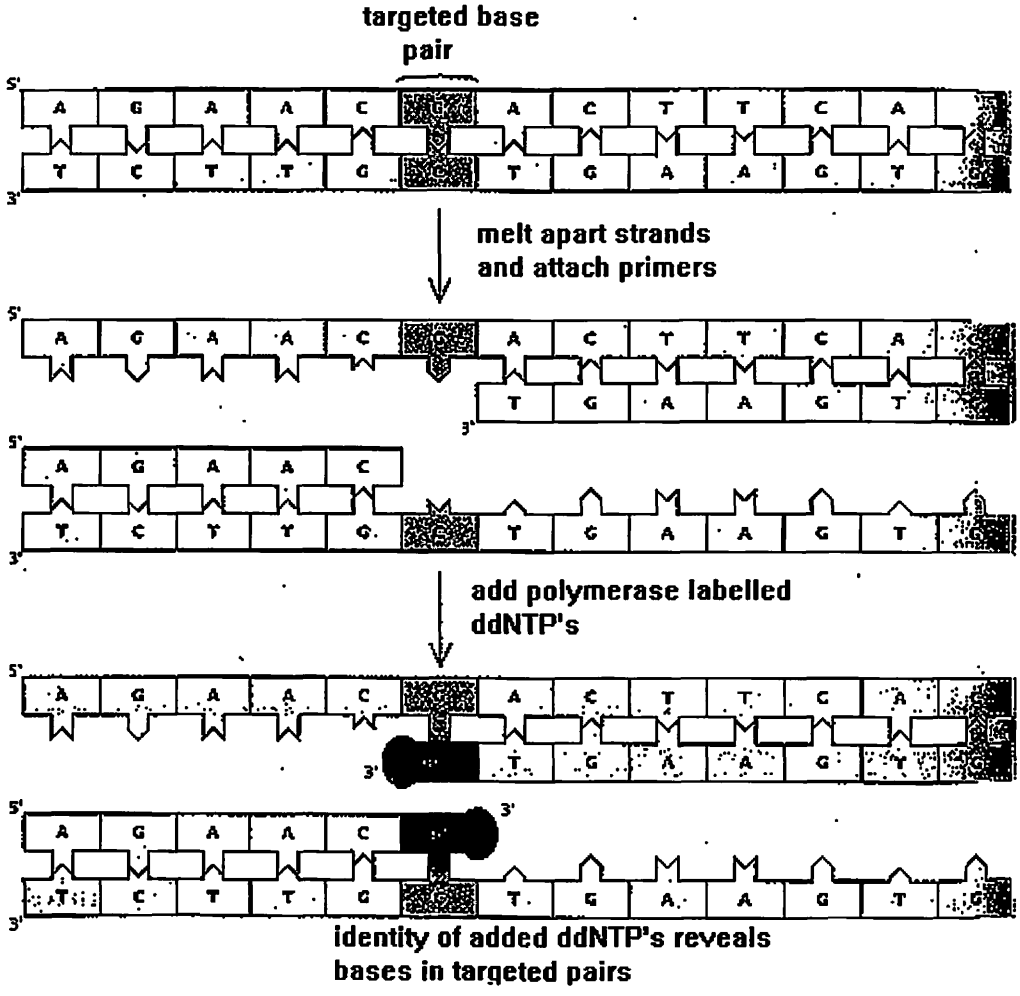
The modern perception of DNA is that it is an easily obtainable object. This misconception is probably a result of the casual discussion of DNA in science fiction, but nothing could be further from the truth. In practice, it is

difficult to obtain particular genes from natural DNA. For one, there is an inherent instability in any polymer. In the absence of additional packaging, the mildest of shearing forces would fragment naked DNA into random sizes which is what occurs in biological artifacts such as mammoth bones. The stability of DNA arises after it is packaged, coiled and then supercoiled in association with various proteins. The resulting structure is exceptionally organized and remarkably stable. The relative instability of the naked molecule, and the fact that any attempt at working with DNA fragments it into pieces of varying sizes, made it difficult to study genes. However in the 1970's, enzymes known as restriction endonucleases were discovered. Restriction enzymes cut DNA at specific sites and made it possible to obtain homologous fragments that were much more identifiable; making it easier to isolate a particular gene. Also at the time, oligonucleotide biochemistry had developed to the point where short nucleotides with specifically ordered bases, could be synthesized under optimum conditions. There nucleotide primers would bind a complementary sequence of DNA and act as a probe.

On that Friday night in 1983, Mullis was driving to Mendocino county with a chemist friend. On that trip he started contemplating an experiment to test his proposed DNA sequencing idea. His plans were straight forward. First he would separate a DNA target into single strands by heating it. Then he would hybridize an oligonucleotide to a complementary sequence on one of the strands. He would place portions of this DNA mixture into four

different tubes. Each tube would contain all four types of dNTP, but in each tube a different type of ddNTP would be radioactively labeled. Next he would add DNA polymerase, which would extend the hybridized oligonucleotides in each tube until a ddNTP was incorporated into the growing chain. By electrophoresis he could separate the extended oligonucleotide from the residual ddNTP's; by identifying which radioactively labeled ddNTP had been incorporated into the oligonucleotide. Since dideoxy nucleotides are chain terminating, he could determine the corresponding complementary base in the target strand. Simple. (Mullis et. al., 1990) (fig.1).

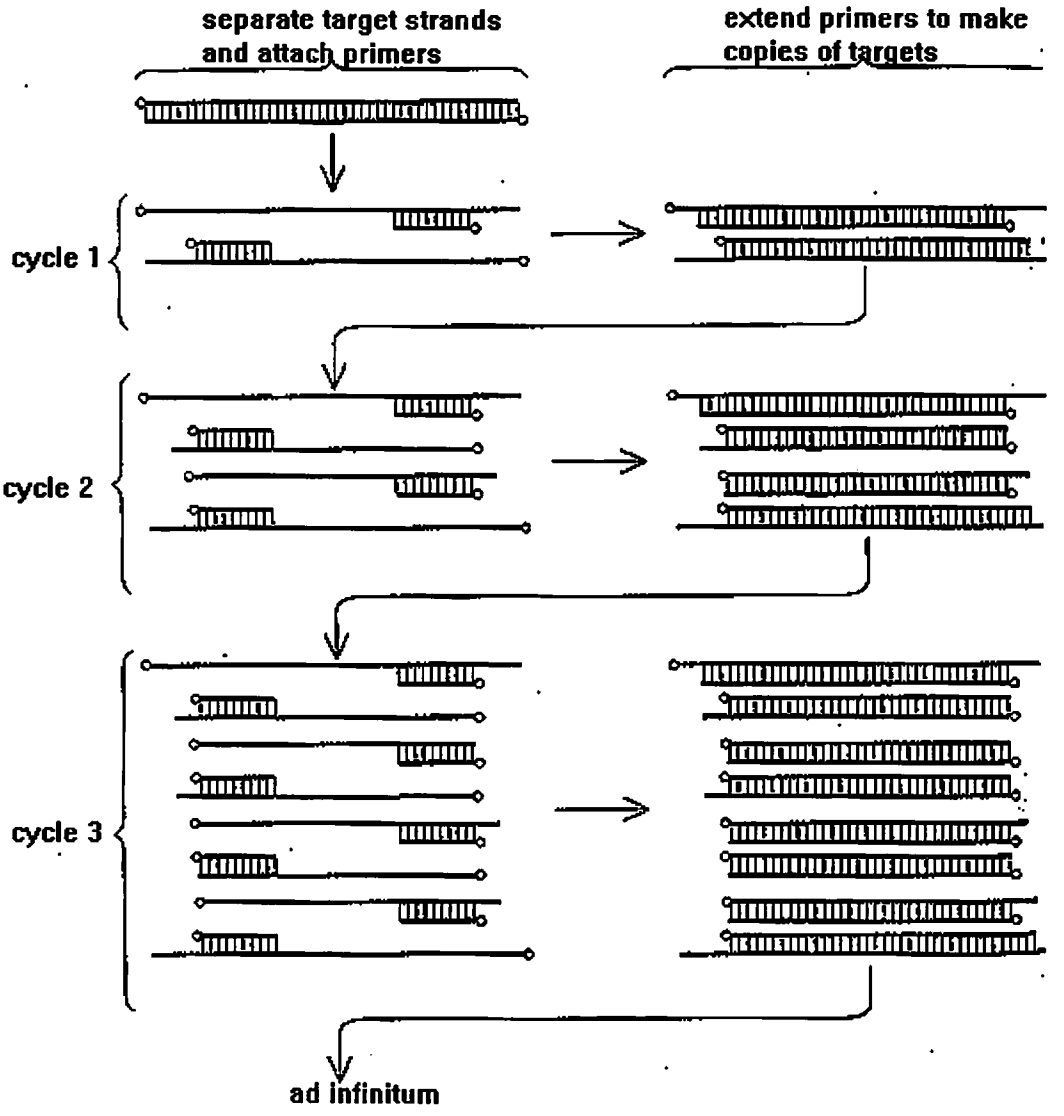
While driving through the coastal range around Cloverdale, Mullis decided to modify his experiment by adding two primers instead of one. In addition, varying the oligonucleotide sizes would allow him to distinguish the oligonucleotides from each other and by directing each of the primers to a particular strand he could get complementary sequence information about each strand. An internal control at no extra inconvenience (Mullis et. al., 1990). However there was a problem. DNA samples contain stray nucleotides that often add themselves to the three prime end of the primer. In his experiment these stray nucleotides would introduce themselves before his ddNTP's thus creating inaccurate results. He originally considered using alkaline phosphatase to cleave the reactive phosphates of the nucleotide triphosphates rendering them inert. However the same phosphatase would



To determine the identity of a targeted base pair in a piece of DNA, Mullis hoped to apply a variation on a tecnique called dideoxy sequencing. First two primers would be bound to the apposing strand in the DNA at sites flanking the targeted pair. DNA polymerase and dideoxynucleotide triphosphate (ddNTP's) sould then be added to the mixture, which would allow each of the primers to be extended by only one base. The identity of the added ddNTP bases would reveal what the complementary targeted bases were. The technique could work with only one primer, but the use of this experiment led Mullis to the polymerase chain reaction.

also destroy his ddNTP's if even the smallest amount was left in his tube when he added them. At the time he wasn't aware that there were ways of inactivating this particular enzyme such as heating in the absence of zinc, so he continued to search for another way to deal with his problem. He theorized that by running a mock trial with all the necessary reagents except the ddNTP's, the polymerase would use up all the stray nucleotides incorporating them into the extending polymer. Then by heating the sample he could separate the strands and the extended nucleotides would be in the sample but at a far less concentration than the unextended nucleotides. At this point any primers would most likely, hybridize with the unextended target DNA and his ddNTP's would then be incorporated at the appropriate sites. However, it still bothered him that there would be oligonucleotides extended by the mock reaction in the sample that could potentially interfere with his experiment. Furthermore, what if the sequences were extended beyond a single base. Adding enough bases could possibly create another binding site for the primers and that would really cause trouble. Or would it. "Suddenly [he] was jolted by a realization: the strands of DNA in the target and the extended oligonucleotide would have the same base sequence. In affect, the mock reaction would have doubled the number of DNA targets in the sample." Ureka. (fig. 2)

Excited about his discovery he pulled over his car and pulled out a pencil and a piece of paper to calculate how many copies this reaction could



Polymerse Chain Reaction is a cyclic process with each cycle, the number of DNA targets doubles. The strands in each targeted DNA duplex are separated by heating and then cooled to allow primers to bind to them. Next, DNA polymerase extend the primers by adding nucleotides to them. In this way duplicates fo the original DNA targets are produced.

create. Immediately he started running powers of two in his head: 2, 4, 8, 16, 32....while remembering vaguely that two to the tenth power is about 1000. He had to check this calculation. He resumed driving for about a mile when he realized another added benefit of this process. The length of the exponentially accumulating DNA strands would be fixed because there ends would be sharply defined by the 5' ends of their oligonucleotide primers. He could also replicate larger fragments of the DNA sample by designing primers that hybridize further apart on it. The fragments would always be discrete entities of specific length (Mullis et. al., 1990).

The next morning all Mullis could think of is someone else must have tried this! "Thousands of investigators had, for various reasons, extended single oligonucleotides with polymerase; surly someone would have noticed the possibility of a polymerase chain reaction. (Mullis et. al., 1990)". When Mullis went back to work the following Monday, he had a search run on DNA polymerase and looked for anything resembling the polymerase chain reaction. Nothing. For the next few weeks he contemplated his new idea, its implications and how to test this polymerase chain reaction. He began discussing it with various colleges who really weren't to impressed. Mullis had a reputation for having very off the wall ideas.

Months had passed before he was able to test his hypothesis. He had spent those months working out details such as what buffer solutions to use, how much to heat and cool the mixtures and so on. Mullis made his

decisions primarily on educated guesses and with the help of papers published on DNA techniques, particularly Kornberg's early paper on DNA polymerase. He selected a 25 base pair target fragment of a plasmid and two oligonucleotide primers that were 11 and 13 bases long, respectively. When everything was ready, He ran his favorite kind of experiment: one involving a single test tube and producing a yes or no answer. Would the PCR amplify the DNA sequence he selected? The answer was yes (Mullis et. al., 1990).

Excited about his results, Mullis looked for someone to share his results with. Working late that evening was a patent agent for the Cetus corporation, Albert Halluin. Mullis began to describe the PCR and very quickly Halluin became enthused. PCR had been described to over 100 people but it took a patent agent to agree with Mullis and see its significance. He was so enthused he wanted to see the still wet, autoradiogram that held the data that Mullis's conclusions were based on. Excited about what he saw, Halluin suggested that he get started working on the patent disclosure immediately.

In the spring at the annual Cetus Scientific meeting, Mullis presented a poster describing his PCR. At first no one seemed interested but Mullis new what he had. Finally he noticed Josh Lederberg the president of Rockefeller University and managed to convince him to consider his idea. " Josh looked the poster over carefully and then turned his enormous head, the head of a

Nobel-laureat, the head that had deduced in 1946 that bacteria could have sex. "Does it work?" He seemed amused." (Mullis et. al., 1990). They talked for a long time. Lederberg began to discuss a conversation that he and Kornberg, the man who discovered DNA polymerase, had 20 years ago on the notion that DNA polymerase could somehow be harnessed to make large quantities of DNA. However, after a couple of experiments they gave up. "I think that Josh, after seeing the utter simplicity of the PCR, was perhaps the first person to feel what is now an almost universal response to it among molecular biologists and other DNA workers: " Why didn't I think of that?" And nobody really knows why, surely I don't . I just ran into it one night."(Mullis et. al., 1990).

Development:

Today many of the technical problelems with PCR have been worked out. Originally PCR was done using a fresh aliquot of Klenow fragment of E coli polymerase 1. However, during each cycle, the polymerase was denatured and had to be readded. The introduction of a DNA polymerase from *Thermus aquaticus (Taq),* a thermophile found in hot springs, eliminated this problem and facilitated automation of the PCR (Saiki et. al., 1988). " Since this heat resistant polymerase is relatively unaffected by the denaturation step, it does not need to be replenished at each cycle. The

modification not only simplifies the procedure, making it amenable to automation, it also substantially improves the overall performance of the reaction by increasing the specificity, yield, sensitivity and length of targets that can be amplified (Saiki et. al., 1988). However, there are some problems with Taq polymerase. Taq polymerase lacks the 3'-5' exonuclease proofreading activity that is present in other polymerases. Saiki estimated the error rate of the Taq polymerase as 2^{-4} which is higher than 2^{-6} error rate of most other DNA polymerases. Using appropriate negative controls or switching to other DNA polymerases with higher fidelity tend to alevate this problem. Another problem with Taq polymerase is that it can self anneal or nonspecifically anneal to improper DNA sequences at room temperature. Under non stringent conditions, products can be generated that arise from annealing of the primer to target DNA locations of low complementary. The result is new template or "tagged" DNA that has the potential of competing with the original polymerization reaction. There are three common ways of dealing with this including what is called the "Hot Start method". The "Hot Start" method requires denaturation of the template and annealing of the primers before Taq polymerase is added to the reaction. This provides a dramatic improvement in specificity in many cases (Chou et. al., 1992). The second method entails cooling all components of the reaction mixture to 0° prior to mixing. This also decreases the likelihood of misprimed products and primer dimerization. Reversible inhibition of Taq polymerase by TaqStart

antibody is the most convenient and effective way of decreasing extension of misprimed products and primer dimerization (Kellog et. al., 1994).

Deoxynucleotide triphosphates (dNTP) concentration are also very important to the overall efficiency of the PCR. It may be tempting to increase the concentration of dNTP's in your reaction in an effort to increase the overall efficiency of amplification, however, this is often disasterous. dNTP's have a tendency to chelate and bind magnesium which could result in destabilization of the DNA. dNTP's in miilimolar concentrations have also been shown to specifically inhibit Taq polymerase (Gelfand et. al., 1989).

Regulation of thermal cycling perimeters are also very important to the overall efficiency of the PCR. Each step requires a minimal amount of time to be effective, since to much time can be both wasteful and deleterious to the DNA polymerase (*). The standard time allotment for the denaturation step is 60 sec. This insures that the tube spends enough time in the water bath to adequately heat the all the DNA to 94° (Gelfand et. al., 1989) Annealing is a much more flexible step in the PCR process. Primers with relatively low GC content (<50%) may require temperatures lower than 55° for full annealing. For primers with high GC content, higher annealing temperatures may be used (Kramer et. al, 1997). Optimum extension temperature is approximately 74°, close enough to optimal Taq polymerase temperatures and yet low enough to prevent the primers from falling off DNA (Gelfand et. al., 1989).

Applications:

Since 1986, PCR amplification has been used in a wide variety of applications. It has found use in Forensics, Archeology, Paleontology, gene expression, disease detection, gene therapy, gene mapping and sequencing, phylogeny analysis as well as many other applications.

The quantitation of rare DNA's by PCR has been one of the most interesting applications of PCR. The most noticeable of these applications is in the forensic sciences. Before PCR, the standard genetic means of analyzing a crime scene was done using typing of variable number tandem repeat (VNTR) loci by restriction fragment length polymorphism (RFLP) analysis via southern blotting. However, this approach had certain limitations. For one, at least 50 nanograms of high molecular weight DNA is required for RFLP analysis. Second, radioactively labeled probes are required to increase the sensitivity of detection. Third, the VNTR-RFLP approach is very time consuming and laborious. Finally, RFLP sometimes cannot resolve unequivocally, the alleles of the VNTR loci. (Budowle et. al, 1994). Recently, a new approach in forensic testing has been developed utilizing PCR based assays. There are three main analytical methods that have been developed;

Dot blot assays using allele specific oligonucleotide probes, electrophoresis to resolve size variants and sequencing. The advantages of PCR based assays are increased sensitivity, decreased assay times, and no need for isotopically labeled probes.

Another application of PCR is in Archeology. "[A] sense of being "time trapped" has led to the desire among molecular evolutionist to attempt to do what paleontologists do, go back in time and directly approach the ancestor DNA. The advent of the polymerase chain reaction has made this possible and thus opened up a new field of study, molecular archeology. (Hoss et. al, 1994)." Before PCR, immunological studies were done using antibodies that detect epitope variations in proteins such as albumin and Alpha keratin. Then based on their percent homology, each particular species studied was placed somewhere in the phylogenetic tree. However, immunological studies of ancient proteins and carbohydrates remains are susceptible to a number of pitfalls due to chemical modifications and degradation's of the antigens, as well as the existence of related antigens on microorganisms giving rise to false positive reactions (Flaherty and Haigh, 1986). The advent of PCR shines new light on the problem of recreating the past. PCR amplifies particular genes that could then be separated from the vast excess of damages molecules. This amplification product could then be used to give an accurate DNA sequence. Furthermore the PCR results are

repeatable allowing for additional experiments designed to eliminate any further errors in the genetic analysis.

Gene expression analysis by Reverse Transcriptase polymerase chain reaction (RT-PCR) is also another means of quanititating rare DNA's via PCR. This method is a rapid and accurate way of qualitating and quantitating mRNA for transcriptional analysis. This approach is vastly superior to past methods as a result of its ability to detect transcripts that have a short half life, low rate of transcription or are present in a low number in cells. RT-PCR has advanced our understanding of gene expression considerably. It has improved our knowledge of many biological systems such as vertebrate embryogenesis as well as many other examples that relate to development, differention, tissue specificity and pathology.

Quantitation of rare DNA's by PCR has also been applied to diagnostics. The accumulation of genetic alterations in critical genes, the oncogenes and tumor suppressing genes, has become the accepted cause of cancer. Detection and characterization of single point mutations in oncogenes and anti oncogenes by assays utilizing PCR, has become common place in cancer diagnostics. Another obvious utilization of quantitation techniques using PCR, is in the detection of RNA and DNA species that are specific to an infectious organism. PCR has also been shown to allow the analysis, at the molecular level, of the progression of infectious diseases and in turn providing a means of assessing therapy effectiveness. PCR also has a

remarkable ability to detect one marked cell among a million unmarked cells allowing for direct gene isolation and sequencing from cellular DNA. PCR was therefore essential to the development of gene therapy, by permitting unambiguous detection of genetically engineered cells. Transfer of an adequate amount of the genetically engineered cells to the patient can only take place after the genetically engineered cells have been adequately identified.

PCR has also spawned advancements in sequencing. The original chemical method deduced by Maxam and Gilbert in 1977 involved using hydrazine, dimethyl sulfate (DMS) or formic acid to cleave specific base pairs. DMS methylates the 7^{th} nitrogen in guanine displacing the modified guanine from the sugar. Formic acid weakens A and G glycosidic bonds specifically cleaving the arganine and guanine glycosidic bond. The thymine and cytosine glycosidic bond is broken by hydrazine in the presence of NaCl. By putting DMS in one tube, formic acid and hydrazine in another and hydrazine alone in yet another tube, all in the presence of NaCl and your DNA sample, you can generate fragments of DNA that coincide with the broken glycosidic bonds. Electrophoresing the four samples in separate lanes allows you to sequence the gene(Kramer et. al,1997). The problem with the Sanger method is that the chemicals are highly hazardous. The second problem is that the reagents have to be continually readded in order for the reaction to continue. Furthermore, the sequencing was sloppy and the bands in the gel

varied in florescence intensity. The dideoxy method (Sanger) utilizes PCR to generate a large amount of single stranded DNA template. There are four separate dideoxynucleotides one for each base. One of each dideoxy is added to a tube along with DNA polymerase, a portion of the DNA sample and other polymerase reagents. Under these conditions, the primers are extended until the dideoxy is incorporated causing termination. It is the series of these terminations that creates fragments of DNA that are specific to the dideoxy added or the base termination that resulted from the addition of the dideoxy. These fragments can then be separated by gel electrophoresis and the DNA sequence read. Originally, the enymatic method was also difficult to read as a result of the inherent problems with using DNA polymerase 1 (*Ecoli*). However, with the addition of Taq polymerase, the enzymatic method of DNA sequencing became super clean and has become the method of choice for DNA sequencing.

In addition to gene sequencing, several approaches to genetic mapping and phylogenetic analysis using PCR have been developed. Prior to the invention of PCR, phylogenetic analysis required cloning and was very labor intensive. "PCR allows for the direct amplification and sequencing of interesting sequences without the need of cloning. With these sequences in hand, geneticists can compare the amplified sequences of various species and derive phylogenetic and population genetics information. They can also use the amplified sequences to probe DNA for gene mapping.

PCR has also been shown to be useful in areas other than biology and medical diagnostics. Individual use of DNA as a commercial tag at the sub microscopic level has begun and is becoming an exciting new application of PCR amplified DNA. There are at least three reasons to use DNA as a commercial tag; to determine the source of the product, monitor product distribution and aid in the ultimate detection of the product.(Kramer et. al, 1997). PCR amplified DNA has also been suggested for identification of counterfeit money. One method of trying to prevent counterfeiting money is to use DNA as a identifiable tag that cannot be forged or removed(Kramer et. al, 1997). The possibility of using PCR and DNA as a taggant for hazardous substances has also been suggested.

Dr. Conway's Laboratory

Agaricus bisporus is a white button mushroom cultivated for its use in the food industry. Every year crops of this mushroom are damaged due to infection by a pathogenic agent called the *La France Virus*. The overall goal of the lab is to create a monoclonal antibody to the mushroom pathogen, the *La France Virus*. This antibody will then be utilized in the development of an assay system designed to screen out spores that are contaminated with this pathogen.

The first step is to create a cDNA copy of the *La France Virus* dsRNA. Typically, cDNA kits such as the cDNA cycle kit for reverse transcriptase PCR from *Invitrogen*, use reverse transcriptase to generate high yields of cDNA for use in PCR amplification. Once cDNA copies of the selected gene have been obtained, PCR amplification is done under varying buffer and ion concentrations. This PCR optimization is used to test a wide variety of reaction conditions in order to identify the conditions most suitable to the primers being used and their template sequences.

The next step is to ligate the PCR amplified DNA copies of the selected gene into a phage, transfect the competent bacteria with the ligated vector and then isolate the colonies of bacteria properly transfected. Most transfection systems provide the scientist with vectors that contain genes for antibiotic resistance as well as additional genes flanking the multiple cloning region. The antibiotic resistance genes as well as the genes flanking the multiple coding region, are specifically designed to provide a means of isolating the properly transfected bacterial colonies in culture. Our system, the PgemT

system, uses the T7RNA and SP6 RNA polymerase promotors within the α-peptide coding region of the enzyme β-galactosidase, to flank the multiple coding region and provide a means of isolating the properly transfected bacteria. Proper insertion of the ligated gene within the PgemT vector causes insertional inactivation of the α-peptide allowing recombinant clones to be detected by color screening on indicator plates. The multiple cloning region also contains a number of unique restriction sites allowing for ease of ligation of the selected gene into the vector.

The development of an antibody to a pathogen ultimately initials purification of one of the virus essential proteins for use in monoclonal antibody development. This will be the next step in our labs research.

My work:

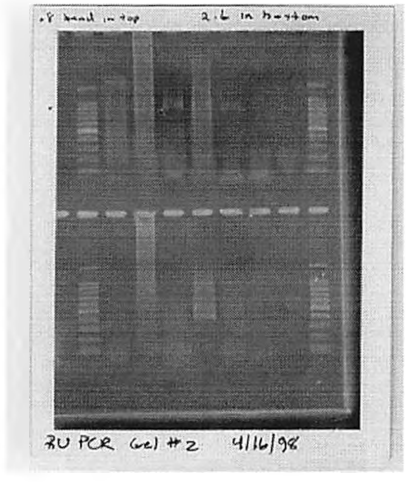
This semester I have been involved in ligation of L3, a gene in the *La France virus*, into the PgemT vector system as well as transfecting Ecoli with our ligated vector. This entire process involves creating a cDNA copy of the L3 RNA viral gene, amplification of the cDNA utilizing PCR, ligating it in to PgemT and then transforming the bacteria. These are the experimental procedures that I followed.

cDNA From viral dsRNA by RT-PCR:

2 ml- La France Virus prep.
6 ml- sterile water
2 ml- MeHgOH - mix by tapping
- spin
- 25° for 5min.
2.5 ml- β-mercaptoethanol- tapp and spin- put on ice
.5 ml- primer 1- 2 min. at 65° then chill
1 ml- Rnase inhibitor
4 ml- RT buffer
1 ml- Dntp's
.5 ml- RT- tapp and spin - 42° for 60 min.
- 95° for 3 min. - spin- on ice.
.5 ml- RT- tapp and spin - 42° for 60 min.
- 95° for 3 min. - spin- on ice.

Amplification of the cDNA via PCR:

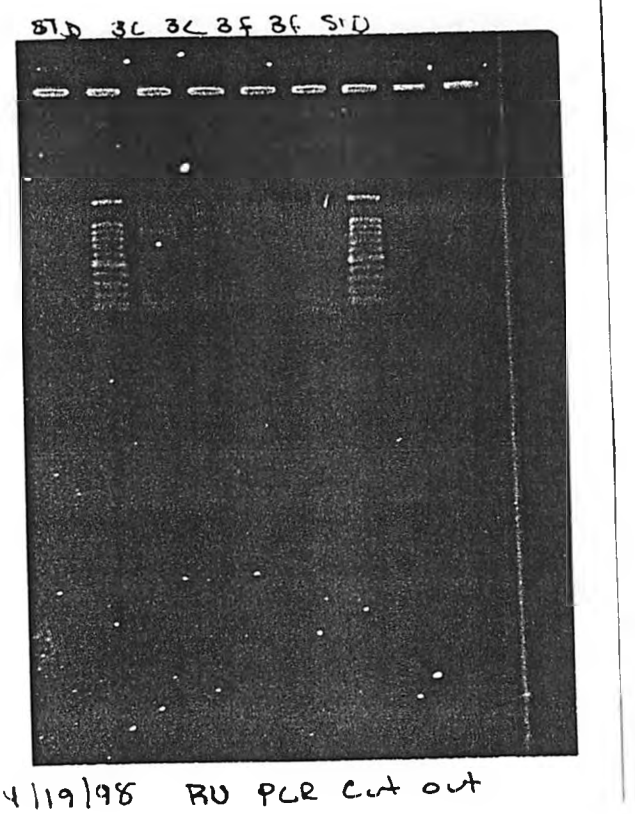
35.7 ml - sterile water
1 ml - primer 1
1 ml - primer 2 or 3
1 ml - cDNA
10 ml - buffer a, b, c, d, f, j, n.
3 ml - Taq polymerase - 80° for 30 sec.
1 ml - Dntp's
overlay with 50 ml - mineral oil
put in cycler
should get ;



*-.8kb band with primers 1 and 3(**top**)*
*-2.6kb band with primers 1 and 2(**bottom**)*

Extraction of bands for Ligation of the L3 cDNA copy into the PgemT vector system:

Run a 1% agar gel.
(4) 6 ml - sample 3c or 3f
1 ml - dye
5 ml - 100 bp ladder
-cut the bands out at approx. 800bp
-place bands in clipped tip containing a filter and then place the loaded tips into tubes.
-centrifuge for 5 min.
-add the rest of the gel to the tubes and centrifuge for 5 min.
- weigh out a micro centrifube tube and then add the DNA suspension (free of the gel) to the weighed tube.
4 ml - sodium acetate
20 ml - ethanol
-precipitate DNA overnight at -15°



4/19/98 RU PCR cut out

Extraction of DNA:

- Spin at 14,000 rpm in centrifuge for 15 min at 5°
- Remove ethanol
- Add 1 milliliter 70% ethanol at 40°
- Spin at 14,000 rpm in centrifuge for 15 min at 4°
- Remove ethanol
-Resuspend in TE buffer (50-100ml)

Ligase Reaction:

- 1ml ligase buffer (10x)
- 1ml PgemT
- 1ml PCR product
- 1ml T4 DNA ligase
- 6ml water
- incubate at 15° overnight

Transformation of Ecoli competent bacteria utilizing the PgemT loaded vector:

- 2ml ligation product (PgemT)
- 50ml Ecoli cells - ice 20 min
- incubate at 42 ° for 50 sec
- on ice 2 min
- 950 milliliters of room temp. circle grow
- incubate 1.5hrs at 37°
- plate 100ml - and incubate overnight

Selection of properly transformed bacteria:

- Transfer white colonies to 2.5 milliliters of broth culture -100mg/ml of ampicillin and circle grow
- Transfer each white colony into its own tube - in this case 5 white colonies
- incubate on shaker at 37° overnight

Isolation of PgemT/L3 DNA:

- Transfer 1.5ml of culture to a tube
- Spin at 14,000 rpm at 4° for 30 sec and then remove the supernatant (media)
- Resuspend pellet in 100ml of solution 1 and vortex
- Add 200ml of solution 2 and mix by inversion- on ice
- Add 150ml solution 3(ice cold) then vortex- ice 5min
- Spin at 14,000 rpm for 5 min at 4° and then transfer the supernatant to a fresh tube
- Add two volumes of absolute ethanol at -21° and then vortex- room temp. 10 min
- Spin at 14,000 for 5 min at 4° remove the supernatant and discard it
- Add 1ml 70% ethanol at 4° then spin at 14,000 rpm for 5 min at 4°
- Remove the ethanol then allow the pellet to dry
- Resuspend the pellet in 50 ml TE buffer+ Rnase

Restriction enzyme analysis:

- 1ml multicore
- .5ml EcoR1
- .5ml NcoR1
- 2ml Sample
- .1ml BSA
- 6.9ml water
- Run a 1% agar gel