Development Application of Polymerase Chain Reaction (PCR)

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Abstract: Polymerase chain reaction (PCR) is an in vitro technique to synthesize large quantities of a given DNA molecule that separates the DNA into two complementary strands, uses a peltier heat pump to quickly heat and cool the DNA and uses the Taq polymerase for the synthesis of DNA. Taq is a bacterium that lives by volcanic sulfer jets at the bottom of the ocean where the temperature is very high. For reverse transcription PCR, primers are short strands of RNA that bind to the target site of DNA molecule. DNA polymerases need to have RNA primers for the beginning of DNA replication. Four dNTPs (deoxyribonucleotide triphosphates) (dGTP, dCTP, dATP and dTTP) are bricks of the DNA molecules and the Taq polymerase uses the dNTPs to build the new DNA molecular chains. The real-time PCR (RT-PCR), also called quantitative RT-PCR (qRT-PCR) or kinetic PCR (kPCR), is a technique used to simultaneously quantify and amplify a DNA molecule, and it is used to determine whether a specific DNA sequence is present in the sample (if it is present, the number of copies in the sample). The procedure of RT-PCR follows the regular PCR procedure, but the DNA is quantified after each round of amplification. Two common methods of quantification are the use of fluorescent dyes that intercalate with double-strand DNA, and modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA. RT-PCR could be combined with reverse transcription PCR to quantify messenger RNA (mRNA) at a particular time for in a particular cell or tissue type. [The Journal of American Science. 2005;1(3):1-47].

Keywords: DNA; polymease chain reaction (PCR); real-time (RT); RNA; science

1. Introduction

Of the 832 Nobel Prizes awarded to Chemistry, Economics, Literature, Physiology or Medicine, Peace and Physics from 1901, three prizes are more important and more valuable than others. The number one valuable Nobel prize is the explanation of the photoelectric effect (Nobel Prize for physics in 1921) and the further formulation of the special and general theories of relativity by Albert Einstein, which revolutionized the science and philosophy on the universe. The number two valuable Nobel prize is the discovery of DNA double helix structure by James Dewey Watson, Francis Harry Compton Crick and Maurice Wilkins (Nobel Prize for Physiology or Medicine, 1962), which made the foundation of modern biochemistry and molecular biology. The number three valuable Nobel Prize is the invention of polymerase chain reaction (PCR) by Kary Banks Mullis (Nobel Prize for Chemistry, 1993), which revolutionized the modern life science technology as the most widely used technique in life science researches and applications.

PCR, abbreviation of polymerase chain reaction, is an in vitro technique to synthesize large quantities of a given DNA molecule. PCR separates the DNA into their two complementary strands, synthesizes new DNA molecules using DNA polymerase and repeats this process very quickly. PCR makes logarithmic amplification of short DNA sequences (100 to 600 bp) within a longer double stranded DNA molecule. PCR was invented in 1985 by Kary Banks Mullis (male, born on December 28 of 1944 in Lenoir North, North Carolina, USA) in Cetus Corporation (Berkeley, California, USA) (Greer, 2006; Mullis, 2006). As a biotechnology company established in Berkeley, California, USA in 1972, Cetus Corporation was the orginal owner of PCR patent and the patent was sold to Hoffmann-La Roche Inc. in 1991. Dr. Kary Banks Mullis awarded Norbel Prize of chemistry for the invention of PCR in 1993, only 8 years after the invention of PCR. As a fast gene detection, PCR technique has revolutionized many aspects of life sciences, such as the diagnosis of genetic defects, the detection of the AIDS virus in human cells, criminologist applications and genetic researches, etc. Let's thank and remember Dr. Mullis for the invention of PCR when we do PCR.

PCR uses a pair of primers (about 20 bp each), that are complementary to a specific sequence on each of the two strands of the target DNA. These primers are extended by a DNA polymerase and the sequence of the new DNA pieces matches the sequence of the template followed the primer. After the new DNA synthesized, the same primers will be released and used again. This let the DNA make a logarithmic amplification. Since DNA amplification must be processed under the single

strand condition, it needs high temperature to separate the double strand DNA in each round of the amplification process. The milestone of DNA amplification exploring is the discovery of a thermo-stable DNA polymerase that is isolated from *Thermus aquaticus* (Taq), a bacterium growing in hot pools near volcanic vent. The thermo-stable DNA polymerase comes from Taq and is called Taq polymerase, which composes the core component of the PCR technique. For PCR, it is not necessary to add new polymerase in every round of amplification. After some rounds of amplification (about 40), the PCR product is abundant enough to be detected with ethidium bromide stain and it can be analyzed on an agarose gel. In order to measure messenger RNA (mRNA), the PCR is extended to use reverse transcriptase to convert mRNA into complementary DNA (cDNA). In many cases this method has been used to measure the levels of a particular mRNA (quantitative) under different condition. Reverse transcriptase PCR analysis of mRNA is often abbreviated as "RT-PCR", which is unfortunate as it can be confused with "real-time PCR" that also abbreviated as RT-PCR (Abdul-Careem, 2006). In this paper the RT-PCR represents real-time PCR.

Traditionally, PCR uses a peltier heat pump to quickly heat and cool the DNA strands and uses the Taq polymerase for the synthesis of DNA molecules. Taq, the abbreviation of *Thermus aquaticus,* is a bacterium that lives in volcanic sulfer jets at the bottom of the ocean where the temperature is very high. They can withstand extremely high temperatures, and that is why they are so valuable in PCR. For reverse transcription PCR, primers are short strands of RNA that bind to the target site of DNA molecule. DNA polymerases need to have RNA primers for the beginning of DNA replication. Four dNTPs (deoxyribonucleotide triphosphates) (dGTP, dCTP, dATP and dTTP) are letters of the DNA alphabet and the Taq polymerase uses the dNTPs to build the new DNA molecular chains.

PCR needs to place a very small amount of DNA molecules that contains the target gene into a PCR test tube. A large amount of primer, which matches the certain sequence of the target gene, is also added for the DNA synthesis tubes. These primers find the right sequence in the DNA, and play starting points for DNA synthesis. When the Taq enzyme is added, the loose nucleotides lock into a DNA sequence dictated by the sequence of that target gene located between the two primers.

The test tube is heated, and the DNA's double helix separates into two strands at the high temperature. The DNA sequence of each strand of the helix is opened and as the temperature is lowered the primers automatically bind to their complementary sequences of the DNA molecules. At the same time, the Taq enzyme links the loose nucleotides to the primer and to each of the separated DNA strands in the appropriate sequence. The complete reaction results in two double helices containing the desired portion of the original sequence. The heating and cooling is repeated many times (normally around 40), doubling the number of DNA copies each heating cycle. After 30 to 50 heating cycles are completed a single copy of a piece of DNA can be multiplied to hundreds of millions.

In the early of 1990's, Higuchi et al. made the analysis of PCR kinetics by constructing a system that detected PCR products as they accumulated (Higuchi, et al., 1993). This real-time system included the intercalator ethidium bromide in each amplification reaction, an adapted thermal cycler to irradiate the samples with ultraviolet light, and detection of the resulting fluorescence with a computer-controlled camera. Amplification produced increasing amounts of double-stranded DNA, which binded ethidium bromide, resulting in a fluorescence increase. By plotting the increase in fluorescence versus cycle number, the system produced amplification plots that provide a more complete picture of the PCR than assaying product accumulation after a fixed number of cycles. This technique to measure the accumulation of PCR products in a real time is called real-time PCR abbreviated as RT-PCR, where the real-time is abbreviated as RT and PCR is the abbreviation of polymease chain reaction. As a milestone of the RT-PCR, Higuchi et al. wrote the following in the journal Biotechnology in 1993: "We describe a simple, quantitative assay for any amplifiable DNA sequence that uses a video camera to monitor multiple polymerase chain reactions (PCRs) simultaneously over the course of thermocycling. The video camera detects the accumulation of double-stranded DNA (dsDNA) in each PCR using the increase in the fluorescence of ethidium bromide (EtBr) that results from its binding duplex DNA. The kinetics of fluorescence accumulation during thermocycling are directly related to the starting number of DNA copies. The fewer cycles necessary to produce a detectable fluorescence, the greater the number of target sequences. Results obtained with this approach indicate that a kinetic approach to PCR analysis can quantitate DNA sensitively, selectively and over a large dynamic range. This approach also provides a means of determining the effect of different reaction conditions on the efficacy of the amplification and

so can provide insight into fundamental PCR processes" (Higuchi, et al., 1993).

There are two types of quantification for RT-PCR. One is absolute quantification which requires an input standard curve with series diluted template. Another one is relative quantification which used to determine fold different in input target that do not need a standard curve and is very commonly used for gene expression analysis.

For living cells in a specific time some genes are expressed and some are not, some expressed lower and some expressed higher. When a particular protein is required by a cell or by a body, the gene coding for that protein is activated. The first step to synthesize a protein is to transcribe an mRNA from the gene's DNA sequence. The amount of mRNA produced correlates with the amount of protein eventually synthesised. Measuring the amount of a particular mRNA produced by a given cell or tissue is often easier and more important than measuring the amount of the final protein, as the protein could be in a dynamic status in the cell's living cycle.

Traditionally, mRNA amount can be measured by Northern blot and this method is still used in many by many laboratories to measure mRNA. Northern blot needs larger of mRNA sample, and RT-PCR was developed to measure small amount of mRNA. As the sensitivity is higher for RT-PCR method, it should be careful on the contamination. For RT-PCR, it does not need to measure the concentrations of mRNA or cDNA in a sample before the detection. The other method for RNA measurement is RNase protection assay.

Normal reverse transcriptase PCR is only semi-quantitative because of the insensitivity of ethidium bromide. PCR is the most sensitive method and can discriminate closely related mRNAs. Northern blot and ribonuclease protection assays are the standard methods. And, in situ hybridization is qualitative rather than quantitative. Techniques such as Northern blot and ribonuclease protection assays work very well, but they require more RNA than it is sometimes available. PCR methods are particularly valuable when amounts of RNA are low, since it is more sensitive. In contrast to regular reverse transcriptase PCR that needs the analysis of agarose gels, RT-PCR gives quantitative results. RT-PCR is the relative easy to do and convenience of use compared to some older methods. RT-PCR offers scientists a powerful tool for the quantitation of target nucleic acids.

In U'Ren, et al's studies, a TaqMan allelic-discrimination assay designed around a synonymous single-nucleotide polymorphism was used to genotype Burkholderia pseudomallei and Burkholderia mallei isolates. The assay rapidly identifies and discriminates between these two highly pathogenic bacteria and does not cross-react with genetic near neighbors, such as Burkholderia thailandensis and Burkholderia cepacia (U'Ren, 2005).

RT-PCR offers the ability to monitor the real-time progress of the PCR product via fluorescent detection. The point characterizes this in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. These PCR based fluorescent homogenous assays can be monitored with either labeled hybridization probe (TaqMan, Molecular Beacons) or labeled PCR primer (Amplifluor) and SYBR Green (Applied Biosystems).

PCR has made a revolution for the life science. As Dr. Kary Banks Mullis wrote in *Scientific American*, "Beginning with a single molecule of the genetic material DNA, the PCR can generate 100 billion similar molecules in an afternoon. The reaction is easy to execute. It requires no more than a test tube, a few simple reagents and a source of heat. The DNA sample that one wishes to copy can be pure, or it can be a minute part of an extremely complex mixture of biological materials. The DNA may come from a hospital tissue specimen, from a single human hair, from a drop of dried blood at the scene of a crime, from the tissues of a mummified brain or from a 40,000-year-old wooly mammoth frozen in a glacier" (Mullis, 1990).

2. PCR History

Dr. Kary Banks Mullis invented PCR technique in 1985 while he worked as a chemist at Cetus Corporation, a biotechnology company established in Berkeley, California, USA in 1972. Cetus Corporation had the orginal ownership of PCR patent. Kary Mullis awarded Norbel Prize of chemistry for the invention of PCR in 1993.

Dr. Kary Banks Mullis, male, was born on December 28 of 1944 in Lenoir North, North Carolina, USA, He obtained his bachelors degree in Chemistry in 1966 from the Georgia Institute of Technology and received a PhD in Biochemistry from the University of California at Berkeley in 1972. He then spent seven years of post-doctoral research on Paediatric Cardiology and Pharmaceutical Chemistry at the University of Kansas Medical School. After his period at Kansas Medical School he got a technician position at the Cetus Corporation of Emeryville (in 1978). It was during the time here that he created the idea for PCR.

In 1983, while driving along the Pacific Coast Highway 128 of California in his Honda Civic from San Francisco to his home in La Jolla, California, USA, Kary Mullis was thinking about a simple method of determining a specific nucleotide from along a stretch of DNA. He then, like many great scientists, claimed having a sudden flash of inspirational vision. He had conceived a way to start and stop DNA polymerase action and repeating numerously, a way of exponentially amplifying a DNA sequence in a test tube. Mullis then took his concept to his colleagues at Cetus Company and together they made it work in an experimental system.

This technique was first opened to the world at a conference in 1985 and was widely accepted by the scientific community after then. The enzyme molecule used in PCR was named as *Taq* Polymerase in 1989. In 1989, Cetus got the patent for the PCR technique. By 1991 the use of PCR in laboratories across the world was extremely widespread.

In 1992, Cetus, who own the patent for the technique, underwent a corporate reorganization and sold the patent of PCR and *Taq* polymerase to Hoffmann-La Roche for $300 million. The USA patent for PCR is a national right and it wants that all American universities and companies who wish to use PCR must obtain a licence. Universities and companies in other countries are exempt from this patent and are allowed to use the technique without a licence.

Due to the unprecedented popularity of the technique and its revolutionary impact on life sciences, Kary Mullis was awarded the Nobel Prize for Chemistry in 1993 for the invention of PCR. This award was argued as others thought that the development of the technique was the scientific advance and that merely visualising the concept did not deserve the prize. However, the PCR is really great!

The concept of PCR involved a collaboration of existing techniques. Mullis claimed that his idea of combining them was the inventive step and essentially, the birth of PCR. This is somewhat true, and although the techniques were not new, some invention was required by his colleagues to recombine and integrate the techniques from theory to practice. People argued that PCR only really became a scientific entity once it became an experimental system.

Chiron Corporation made an important contribution to the development of PCR, especially in the human immunodeficiency virus (HIV) diagnosis application of PCR. Chiron Corporation was a multinational biotechnology firm based in Emeryville, California, USA, that was acquired by Novartis International AG on April 30, 2006. It had offices and facilities in eighteen countries on five continents. Chiron's business and research was in three main areas: biopharmaceuticals, vaccines and blood testing. Chiron's vaccines and blood testing units have been combined to form Novartis Vaccines and Diagnostics, while Chiron BioPharmaceuticals will be integrated into Novartis Pharmaceuticals. Chiron was founded in 1981 by professors William Rutter, Edward Penhoet, and Pablo Valenzuela. In 1992, the company's first product, Proleukin, was approved in USA for the treatment of metastatic kidney cancer. On August 27, 2003 two bombs exploded at Chiron's headquarters in Emeryville, California, USA. A group named Revolutionary Cells of the Animal Liberation Brigade sent the email to reporters to claim that it is this organization making the bombing. In 2005, Chiron Corporation’s revenue was $1.921 billion and it had 5,400 employees.

When doing manually, Mullis' PCR was slow and laborious. Therefore, Cetus scientists began looking for ways in which to automate the process. Before the discovery of the thermostable Taq enzyme, scientists needed to add fresh enzyme to each cycle. The first thermocycling machine (Mr. Cycle) was developed by Cetus engineers to address that need to add fresh enzyme to each test tube after the heating and cooling process. The purification of the Taq polymerase resulted in the need for a machine to cycle more rapidly among different temperatures. In 1985, Cetus formed a joint venture with Perkin-Elmer Corporation in Norwalk, Connecticut, USA, and introduced the DNA Thermal Cycler. By 1988, Cetus was receiving numerous inquiries about licensing to perform PCR for commercial diagnostic purposes. On January 15, 1989, Cetus announced an agreement to collaborate with Hoffman-LaRoche on the development and commercialization of *in vitro* human diagnostic products and services based on PCR technology. Roche Molecular Systems Company eventually bought the PCR patent and associated technology from Cetus for three hundred million US dollars.

Dr. Kary Mullis has written that he conceived of PCR while driving along the Pacific Coast Highway 128 of California one night in his Honda Civic car. He was playing in his mind with a new way of analyzing gene mutations when he realized that he had invented a method of amplifying any given DNA sequence. Mullis has stated that it was the psychedelic drug lysergic acid diethylamide (LSD) that helped him to invented PCR technique. "Would I have invented PCR if I hadn't taken LSD?

I seriously doubt it. I could sit on a DNA molecule and watch the polymers go by. I learned that partly on psychedelic drugs", he stated (Oehlert, 2006). LSD is a semisynthetic psychedelic drug. LSD is a powerful drug. A typical single dose of LSD during the 1960s was between 0.2 and 100 ng. Today, a typical single dose of LSD can be as low as 0.025–0.05 ng. The effects of LSD can vary greatly, depending on factors such as previous experiences, state of mind and environment, as well as dose strength. Generally, LSD causes expansion and altered experience of senses, emotions, memories, time, and awareness. In addition, LSD may produce visual effects such as moving geometric patterns, "trails" behind moving objects, and brilliant colors. LSD does not produce hallucinations in the strict sense but instead illusions and vivid daydream-like fantasies, in which ordinary objects and experiences can take on entirely different appearances or meanings. At higher doses it can cause synaesthesia. The drug experience sometimes spurs long-term or even permanent changes in a user's personality and life perspective. The story of the LSD helping the invention of PCR let us see that the drug addict, alcoholic or smoking are not always bad. Human brain is complex, and the stimulating of drug, alcohol and cigarette can hurt and human brain, but can also comfort the brain. This is why the drug, alcohol and cigarette exit so long time – almost companied with the whole human history. And right now, no matter how strong the human society to against the drug, alcohol and cigarette, they are sold and used everywhere. They can induce the scientific revolution like the PCR invention, sometimes.

Dr. Kary Mullis was awarded the Nobel Prize in Chemistry in 1993 for the PCR invention, only 8 years after he and his colleagues at Cetus first reduced his proposal to practice. Mullis's idea was to develop a process by which DNA could be artificially multiplied through repeated cycles of duplication driven by an enzyme called DNA polymerase.

DNA polymerase occurs naturally in living organisms, where it functions to duplicate DNA when cells divide in mitosis and meiosis. Polymerase works by binding to a single DNA strand and creating the complementary strand. In Mullis's original process, the enzyme was used in vitro. The double-stranded DNA was separated into two single strands by heating it to 94°C. At this temperature, however, the DNA polymerase used at the time was destroyed, so the enzyme had to be replenished after the heating stage of each cycle. Mullis's original procedure was very inefficient, since it required a great deal of time, large amounts of DNA polymerase, and continual attention throughout the process.

Later, this original PCR process was greatly improved by the use of DNA polymerase taken from thermophilic bacteria grown in geysers at a temperature of over 110°C. The DNA polymerase taken from these organisms is stable at high temperatures and, when used in PCR, does not break down when the mixture was heated to separate the DNA strands. Since there was no longer a need to add new DNA polymerase for each cycle, the process of copying a given DNA strand could be simplified and automated.

One of the first thermostable DNA polymerases was obtained from *Thermus aquaticus* and was called Taq enzyme. Taq polymerase is widely used in current PCR practice. A disadvantage of Taq is that it sometimes makes mistakes when copying DNA, leading to mutations in the DNA sequence, since it lacks 3'→5' proofreading exonuclease activity. Polymerases such as *Pwo* or *Pfu,* obtained from *Archaea,* have *proofreading mechanisms* (mechanisms that check for errors) and can significantly reduce the number of mutations that occur in the copied DNA sequence. However these enzymes polymerize DNA at a much slower rate than Taq. Combinations of both *Taq* and *Pfu* are available nowadays that provide both high processivity and high fidelity.

PCR has been performed on DNA larger than 10,000 bp, however the average PCR is only 200-2000 bp of DNA. The problem with long PCR is that there is a balance between accuracy and processivity of the enzyme. Usually, the longer the fragment, the greater the probability of errors.

3. PCR Patent

The PCR technique was patented by Cetus Corporation, where Mullis worked when he invented the technique in 1983. The Taq polymerase enzyme is also covered by the patents. There have been several high-profile lawsuits related to the PCR technique patents, including an unsuccessful lawsuit brought by DuPont (founded in July 1802 as a gun powder plant by Eleuthère Irénée du Pont, Brandywine Creek, Delaware, USA). The pharmaceutical company Hoffmann-La Roche (Founded in 1896 by Fritz Hoffmann-La Roche, Nutley, New Jersey, USA) purchased the rights to the PCR patents in 1992.

A related patent battle over the Taq polymerase enzyme is still ongoing in several jurisdictions around the world between Roche and Promega (Founded 1978, Madison, Wisconsin, USA). However, the PCR and Taq polymerase patents expire in 2006.

Since March 28, 2005, it no longer needs a license to practice the basic PCR amplification process, which was covered by USA Patents 4,683,195, 4,683,202 and 4,965,188. This much-anticipated opportunity has opened the door for an influx of suppliers hoping to provide Taq DNA polymerase without the controlled by of a license to people with a cheater price.

4. Brief List for PCR Development

1983 Kary Banks Mullis invented PCR technique at Cetus Corporation, California.

1985 Cetus Corporation filed first PCR patent application.

- First publication was published of PCR by Cetus Corporation, which first described the PCR process, amplification of human β-globin genes and application to clinical diagnosis.

1986 Purified *Taq* polymerase was first used in PCR as a replacement to Klenow.

- First forensic practice of DNA typing (HLA-DQA locus) by PCR was used in USA.

1987 Cetus obtained patents for PCR.

1988 PerkinElmer created automated thermal cycler.

- First post-conviction review was made using PCR on a forensic specimen.

1989 Science declared *Taq* polymerase as "molecule of the year".

- Hoffmann-La Roche Inc. and Cetus agreed to begin joint development of diagnostic applications for PCR.

1990 First forensic PCR kit was introduced for HLA-DQA, a polymorphic genetic locus useful for human individual identification.

- Drs. H. Erlich and K. Mullis received the Biochemical Analysis Award from the German Society of Clinical Chemistry.
- Dr. D. Gelfand and Ms. S. Stoffel were named Distinguished Inventors for purifying *Taq* DNA polymerase.
- First simultaneous amplification and detection of specific DNA sequences were made using a fluorescent DNA-binding dye, laying the foundation for future RT-PCR (TaqMan tests).

1991 RT-PCR was developed using a single thermostable polymerase, r*Tth*, facilitating diagnostic tests for RNA viruses.

- Dr. H. Erlich received the Advanced Technology in Biotechnology Milano Award from the International Federation of Clinical Chemistry.
- Hoffmann-La Roche Inc. acquired worldwide rights and patents to PCR.
- Roche Molecular Systems, Inc. was founded exclusively to develop diagnostic and other tests utilizing PCR technology.

1992 AMPLICOR Chlamydia trachomatis Test and AMPLICOR HIV-1 MONITOR Test were introduced outside of the USA.

1993 **D**r. Kary Banks Mullis obtained Nobel Prize in Chemistry for conceiving PCR technology.

- AMPLICOR CT Test received 510K clearance by the US Food and Drug Administration (FDA) and launches in the USA, making it the first FDA-cleared PCR test.
- AMPLICOR HCV MONITOR Test was introduced outside of the USA.

1994 *rTth* EZ RT-PCR research kit appeared.

- The first US patents was issued to RMS inventors for thermostable reverse transcriptase.

1995 COBAS AMPLICOR analyzer, the first automated system for routine diagnostic PCR, launched outside of USA.

- AMPLICOR HIV-1 MONITOR Test and AMPLICOR HCV MONITOR Test, the

first standardized "quantitative" PCR kits, launched outside of USA.

- Introduction of AmpliTaq DNA polymerase FS (the enzyme that sequenced the Human Genome) appeared.

1996 Introduction of an internal control in AMPLICOR CT/NG Test appeared.

- FDA approved AMPLICOR HIV-1 MONITOR Test.

1997 FDA cleared COBAS AMPLICOR Analyzer and the product was launched in USA.

1998 FDA cleared COBAS AMPLICOR Analyzer for clinical use.

- FDA approved COBAS AMPLICOR Chlamydia trachomatis Detection Test.
- Dynal launched DQB1-25, HLA B-56 and HLA A-35 HLA-typing kits for use in transplantation tissue typing.

1999 FDA approved AMPLICOR HIV-1 MONITOR UltraSensitive Test and cleared COBAS AMPLICOR CT/NG Test.

- US blood centers implemented nucleic acid technology (NAT) testing for HCV and HIV using COBAS AmpliScreen HIV-1 Test and COBAS AmpliScreen HCV Test under an Investigational New Drug (IND) application.
- US patent awarded to RMD inventors for thermostable ribonucleotide incorporating "designer" DNA polymerase.
- US patent awarded to RMD inventors for the method of monitoring nucleic acid amplification reactions using a dye-based, probeless process of simultaneous PCR amplification, detection and quantitation (RT-PCR or kinetic PCR).
- LightCycler® TeloTAGGG hTERT Quantification Kit launched.
- Japanese Red Cross Society implemented NAT testing to screen 100% of donated blood for HIV, HCV and HBV using AmpliNAT MPX system.

2000 **US** National Human Genome Research Institute (NHGRI) of the National Institutes of Health (NIH) awarded three-year, $1.2 million grant for development of SNP genotyping program using kinetic thermocycler technology to Drs. Gary Peltz, Roche Bioscience (now Roche Palo Alto) and Russell Higuchi of RMD.

- Dr. Henry Erlich received Association for Molecular Pathology's "Award for Excellence" and National Institute of Justice "Profiles in DNA Courage" Award.
- Agreement reached between Roche and Chiron regarding a broad patent license for probe-based clinical diagnostics for HCV and HIV-1.
- LightCycler® TeloTAGGG hTR Quantification Kit launched.
- Dynal launched HLA C-34 typing kit for tissue typing.
- Roche's LinearArray CF Gold, a cystic Fibrosis mutation-detection product, was launched as an Analyte Specific Reagent in USA.

2001 US patent awarded to RMD inventors for a fiber-optic-based PCR device to simultaneously amplify, detect and quantitate nucleic acids (RT-PCR or kinetic PCR).

- COBAS AmpliPrep System launched outside of USA for research use.
- COBAS AmpliScreen HCV and HIV Test kits launched outside of USA for use in blood screening.
- Roche and Chiron Corporation reached agreement on licensing terms for use of HIV-1 and HCV intellectual property for NAT testing to screen blood, plasma and blood products intended for transfusion.
- FDA approved AMPLICOR HCV Test 2.0 and COBAS AMPLICOR HCV Test 2.0.
- TaqManHCV Analyte Specific Reagent launched in USA.

2002 FDA cleared Roche's next generation automated PCR system, the COBAS TaqMan Analyzer, Series 96, for commercial use in USA.

- COBAS AmpliScreen HIV-1 Test, v1.5 received registration in Italy.
- FDA approved AMPLICOR HIV-1 MONITOR Test, version 1.5, a test with the ability to measure HIV-1 RNA down to 50 copies/ml.
- Roche received FDA clearance for the COBAS AmpliScreen System for use in laboratories testing plasma specimens in the blood screening market.
- FDA approved the COBAS AmpliScreen HCV Test, v2.0 and the COBAS AmpliScreen HIV-1 Test, v1.5.
- FDA granted an Investigational New Drug Application (IND) for the COBAS AmpliScreen HBV Test in July 2002, and in August 2002 Roche begins clinical trials of this test.

2003 Roche and deCODE genetics announced that deCODE had identified specific variations within a single gene that confer significant increased risk of osteoporosis using PCR.

- Roche licensed Affymetrix microarray technology in order to develop and commercialize diagnostic products in a broad range of human disease areas, such as cancer, osteoporosis, cardiovascular, metabolic, infectious diseases, and inflammatory diseases.
- Roche received FDA approval for the COBAS AMPLICOR HIV-1 MONITOR TEST, version 1.5, the automated version of the AMPLICOR HIV-1 MONITOR Test, version 1.5.
- Roche Diagnostics received FDA authorization to begin clinical trials for the TaqScreen West Nile Virus Test, the first fully automated nucleic acid system for screening North American blood supplies.
- Roche Diagnostics and Genome Institute of Singapore announce they have formed an agreement to co-develop a SARS detection kit based on Roche's patented PCR.
- Roche launched the AmpliChip CYP450 microarray.

2004 PCR has been widely used in paternity testing.

2005 RT-PCR and qRT-PCR were widely used.

2006 PCR and Taq polymerase patents expire.

- About 1 million articles have been published on PCR up to now.

(Roche Diagnostics, 2006).

5. Main Contributors to PCR Development

The following is the main contributors on the PCR invention, most were/are related to Cetus Corporation, Roche Molecular Systems and Perkin-Elmer Corporation.

1) Norman Arnheim first became interested in the study of medicine in high school, as the result of a summer spent working at a hospital. He received his B.A. (1960) and M.A. (1962) from the University of Rochester of USA, and his Ph.D. (1966) in *Drosophila* genetics from the University of California, Berkeley, California, USA. Currently serving as professor of molecular biology at the University of Southern California, Arnheim formerly worked at Cetus Corporation on PCR. John G. Atwood came to Perkin-Elmer Corporation in November 1948 with a masters' degree in electrical engineering from Columbia University (1948). He currently serves as senior scientist for the biotechnology instrument group.
2) Peter Barrett holds a B.S. in chemistry from Lowell Technological Institute and a Ph.D. in analytical chemistry from Northeastern University, Chicago, Illinois, USA. He joined Perkin-Elmer in 1970 as product specialist in the Instrument Division, was promoted to manager of the Applications Laboratory in 1982, and director of the Laboratory Robotics Department in 1985. In 1988, Barrett was named director of European Marketing and relocated to Italy. In 1989, he moved to Germany to set up the European Sales and Service Center. He returned to the USA in 1990 to serve as division vice-president of Instruments and was named vice-president of the Life Sciences Division in 1991. In 1993, in conjunction with the merger with Applied Biosystems Incorporated, he moved to California to become executive vice-president, Applied Biosystems Division.

3) Joseph L. DiCesare received his Ph.D. in biochemistry from the University of Rhode Island. In 1976, he accepted the position of assistant product line manager at Perkin-Elmer Corporation and was appointed product line manager of the Gas Chromatography division in 1983. In 1987, he was promoted to the position of Research and Development Applications manager of the Biotechnology Division. Henry Anthony Erlich received his B.A. in biochemical sciences from Harvard University in 1965 and his Ph.D. in genetics from University of Washington in 1972. He served as a postdoctoral fellow in the Department of Biology at Princeton University from 1972 to 1975 and in the Department of Medicine at Stanford University from 1975 to 1979. He joined the Cetus Corporation in 1979 and was appointed senior scientist and director of Human Genetics in 1981. After the dissolution of Cetus in 1991, Erlich transferred to Roche Molecular Systems to serve as director of Human Genetics.
4) Fred Faloona began working as a research assistant under the supervising of Kary Mullis at the Cetus Corporation in 1983, just a few years after graduating from high school. He assisted Mullis with the initial development and application of PCR. He followed Mullis to Xytronyx Incorporated in 1986 where he served as a research associate working on DNA and RNA sequencing and further applications of PCR. In 1988, he returned to Cetus as a research assistant where he worked on the application of PCR to the discovery of new retroviruses and he further refined PCR detection techniques. In 1991, Faloona and a partner began Saddle Point System, a small company designing computer hardware and software.
5) David H. Gelfand completed his B.A. in Biology at Brandeis University in 1966. After receiving a Ph.D. in Biology from the University of California, San Diego in 1970, he began to work as an assistant research biochemist at the University of California in San Francisco. He was offered the position of director of Recombinant Molecular Research at Cetus in 1976 and was promoted to vice-president of that division in 1979. He later got positions as vice-president of Scientific Affairs and director of Core Technology, PCR Division, in 1981 and 1988. In 1991, Gelfand also transferred to Roche Molecular Systems to serve as director for the Program in Core Research.
6) Lawrence Allen Haff received his B.S. in Biochemistry from Michigan State University in 1969. After completing his Ph.D. in biochemistry from Cornell University in 1974, Haff served as a research fellow in the biological laboratories of Harvard University. In 1976, he accepted the position of senior research scientist at Pharmacia. He transferred to Millipore Corporation in 1982 to serve as technical research manager developing and supporting high performance separation techniques. He joined the Perkin-Elmer Corporation in 1985 as principle scientist and research manager to help develop the DNA Thermal Cycler.
7) David C. Jones worked as a stress engineer for the Boeing Commercial Aircraft Company, just after receiving his B.S. in mechanical engineering from the University of California-Davis in 1978. In 1980, he joined the Bio-Rad Laboratories designing and developing chromatography instruments. He got the position of mechanical engineer at Cetus Corporation in 1986 to work on thermocycling instrumentation. He also completed an M.B.A. in management from Golden State University in 1988.
8) Elena D. Katz was awarded her M.S. degree in chemistry from Moscow University, Russia. From 1969 to 1972, she studied in the Ph.D. program at the Institute of Physical Chemistry of the Academy of Sciences in Moscow. In 1973, she was appointed associate researcher in the physical chemistry department of Moscow University. After moving to USA, Katz became Senior Staff Scientist at Perkin-Elmer in 1977 working on various multidisciplinary projects utilizing liquid and gas chromatography. From 1985, Katz studied chemistry at University of London for Ph.D. Shirley Kwok became a research associate with the Assay Department of Cetus Corporation after graduating from the University of California, Berkeley, with a degree in microbiology. Kwok was part of a group of researchers devoted to the use of PCR to detect HIV in human cells. Currently, she is a research

investigator for Hoffman-La Roche at Roche Molecular Systems.

9) Richard Leath started with Cetus in 1980, after receiving a M.S. in electrical engineering from Purdue University, Indiana, USA in 1974. Leath developed the machines as Mr. Cycle, and is a senior engineer at Maxwell Labs, Richmond, California, USA, a company which develops particle accelerators.
10) Kary B. Mullis received his B.S. in chemistry from the Georgia Institute of Technology in 1966 and his Ph.D. in biochemistry from the University of California, Berkeley, California, USA in 1972. In 1973, he worked as a postdoctoral fellow in pediatric cardiology at the University of Kansas Medical School. He returned to California in 1977 and worked as a research fellow in pharmaceutical chemistry at University of California, San Francisco to study endorphins and the opiate receptor. In 1979, he began to work as a scientist in the Chemistry Department of Cetus Corporation in 1979 to study oligonucleotide synthesis and chemistry. He moved to the Department of Human Genetics in 1984 to conduct research on DNA technology. In 1986, Mullis worked as a director of Molecular Biology Department at Xytronyx, Inc. to study DNA technology, photochemistry, and photobiology. He left Xytronyx in 1988 and currently works as a private consultant to a variety of companies in life science. Mullis won the Nobel Prize in chemistry in 1993 for the invention of the PCR technique.
11) Lynn H. Pasahow graduated from Stanford University in 1969 and received his law degree from the University of California at Berkeley School of Law in 1972. He worked in McCutchen, Doyle, Brown, and Enersen in 1973, and currently charges companies' intellectual property affair. He had advised clients and handled complex litigation involving patent, copyright, trademark, trade secret, licensing, export-import, noncompetition, and trade regulation disputes, most involving biotechnology, computer hardware and software and other advanced technology products. He led the group of lawyers which successfully obtained a jury verdict upholding Cetus' PCR patents against the Dupont Company challenge.
12) Enrico Picozza began work with Perkin-Elmer in June 1985, shortly after receiving his degree from the University of Connecticut. Currently, he is working as senior technical specialist, and is devoted to specifying, developing, testing and evaluating instrumentation primarily for the PCR market.
13) Riccardo Pigliucci got his B.S. in chemistry in Milan, Italy and studied as a graduate student of the management program at the Northeastern University, Boston, USA. He joined Perkin-Elmer in 1966 and held numerous management positions in analytical instrument operations in Europe as well as in the USA. He was the general manager of the USA Instrument Division in 1989 after serving as director of Worldwide Instrument Marketing since 1985. In 1988, Pigliucci was appointed a sector vice-president in Connecticut Operations. In 1989, he was elected corporate vice-president. Perkin-Elmer Instruments. He became president of the Instrument Group in 1991 and was named senior vice-president of Perkin-Elmer Corporation in 1992. In 1993, he was elected president and chief operating officer. He is also a director of the Corporation.
14) Randall K. Saiki worked as a laboratory technician in the Department of Microbiology at University of Washington for 1 year, just after he got his B.S. in chemistry and biology from the University of Washington in 1978. In 1979, he moved to the Biology Department at Washington University as a lab technician. He joined the Cetus Corporation in late 1979 as a research assistant in the Recombinant DNA Group. In 1981, he was promoted to research associate in the Department of Human Genetics and was named scientist in that department in 1989. Saiki moved to Roche Molecular Systems in 1991 to serve as research investigator in the Department of Human Genetics. Stephen Scharf received a degree in bacteriology from University of California, Davis. He worked at University of California as a biochemist for 4 years until 1980, when he came to Cetus. Scharf was a research associate in the Department of Human Genetics at Cetus at the time PCR was developed. Currently, he serves as senior scientist at Roche Molecular Systems.

15) Donna Marie Seyfried got her B.S. from Lehigh University in microbiology, then worked as a microbiologist for the E.I. Dupont de Nemours Company. Seyfried moved to Perkin-Elmer in 1985. From 1990 to 1993, she served as business director for Biotechnology Instrument Systems. In 1994, she was appointed director of Corporate Business Development and Strategic Planning. She was responsible for managing the development, commercialization, and marketing of the PCR business as part of the Perkin-Elmer Cetus Joint Venture, and the subsequent strategic alliance with Hoffman-LaRoche. She was also involved in the merging of Perkin-Elmer Applied Biosystems.
16) John J. Sninsky got his B.S. from Bates College in 1972 and Ph.D. from Purdue University, West Lafayette, Indiana, USA in 1976. After getting Ph.D., John J. Sninsky started to work as a postdoctoral fellow in the Departments of Genetics and Medicine at the Stanford University School of Medicine. In 1981, he worked as an assistant professor at the Albert Einstein College of Medicine. He joined the Cetus Corporation in 1984 as a senior scientist in the Department of Microbial Genetics. In 1985, he was appointed director of the Diagnostics Program and of the Department of Infectious Diseases. In 1988, he was promoted to senior director of both of those departments. Sninsky transferred to Roche Molecular Systems in 1991 to serve as senior director for research.
17) Robert Watson, who joined Cetus in 1977, is currently functioning as a research investigator with Roche Molecular Systems, working on nucleic acid-based diagnostics.
18) Thomas J. White graduated from John Hopkins University in 1967 with a B.A. in Chemistry. After serving for 4 years as a Peace Corps volunteer in Liberia, he received his Ph.D. in biochemistry from the University of California, Berkeley in 1976. In 1978, he joined the Cetus Corporation as a scientist, and was promoted to director of Molecular and Biological Research and associate director of Research and Development in 1981. He was appointed vice president of Research in 1984. He moved to Roche Diagnostics Research in 1989 to serve as senior director and in 1991 was appointed vice president of Research and Development of Roche Molecular Systems and associate vice president of Hoffman-LaRoche, Incorporated.
19) Joseph Widunas**,** who graduated from the University of Illinois with a degree in engineering in 1975, came to Cetus in 1981 as a sound engineer. Now director of new product development for Colestech Corporation, Hayward, California, he was instrumental in the development of the second Mr. Cycle prototype, "Son of Mr. Cycle."
20) Timothy M. Woudenberg received his B.S. in Chemistry from Purdue University, West Lafayette, Indiana, USA in 1980. He worked as an electronics design engineer for Mulab Incorporated from 1980 to 1982. He served as a teaching and research assistant at Tufts University from 1982 to 1987 and there completed his Ph.D. in Physical Chemistry in 1988. He joined Perkin-Elmer in 1987 as an engineer in the Instrument Division of the Biotechnology Department.

6. Traditional PCR

As a molecular biology technique, PCR replicates DNA enzymatically *in vitro*. Like DNA amplification in living organisms, PCR makes a small amount of the DNA molecule to be amplified exponentially. However, because it is an *in vitro* technique, it can be performed without restrictions on the form of DNA and it can be extensively modified to perform a wide array of genetic manipulations (Kaldosh, 2006).

PCR is commonly used in life science researches for a variety of tasks, such as detection of hereditary diseases, identification of genetic fingerprints, clinical diagnosis of infectious diseases, cloning of genes, paternity testing, and DNA computing, etc. PCR is used to amplify a short, well-defined part of a DNA strand. This can be a single gene, or just a part of a gene. As opposed to living organisms, PCR process can copy only short DNA fragments, usually up to 10 kb. Certain methods can copy fragments up to 47 kb in size, which is still much less than the chromosomal DNA of a eukaryotic cell. A human chromosome contains about 3×10^6 kp.

Two synthetic oligonucleotide primers, which are complementary to two regions of the target DNA (one for each strand) to be amplified, are added to the target DNA, in the presence of excess

deoxynucleotides and Taq polymerase, a heat stable DNA polymerase. In a series of temperature cycles, the target DNA is repeatedly denatured (at around 95^{o}C), annealed to the primers (at around 55^{o}C) and a new strand extended from the primers (at around 72^{o}C). As the new strands themselves act as templates for subsequent cycles, DNA fragments matching both primers are amplified exponentially, rather than linearly.

1) Normally, PCR requires several basic components:

(1) DNA template, which contains the target DNA fragment
(2) Two primers (sense and anti-sense), which determine the beginning and end of the region to be amplified
(3) Taq polymerase (a thermal DNA polymerase), which synthesize DNA for the amplification and can stand for high tem[perature
(4) Four deoxynucleotides-triphosphates (dNTP, i.e. dATP, dTTP, dGTP, dCTP), from which the DNA polymerase builds the new DNA molecules
(5) Buffer, which provides a suitable chemical environment for the DNA amplification
(6) The PCR process is carried out in a thermal cycler. This is a machine that heats and cools the reaction tubes within it to the precise temperature required for each step of the reaction. These machines cost about US$2,000 – US$20,000.

2) Materials for normal running of PCR

(1) Template DNA (genomic, plasmid, cosmid, bacterial/yeast colony, etc.).
(2) Primers (resuspended to a known concentration with sterile TE).
(3) Buffer (usually 10×, normally supplied with Taq polymerase).
(4) $MgCl_2$ (25 mM).
(5) Taq polymerase.
(6) dNTPs (2 mM for each dNTP stock). Store at -20°C.
(7) Sterile distilled water.
(8) Gloves.
(9) PCR machine (cycler).
(10) Aerosol tips.
(11) Ice.

3) The final concentrations of reagents in PCR reactions

(1) **Buffer:** 1×, usually comes as 10× stock.
(2) **dNTPs:** For most general PCR, the final concentration is 0.2 mM.
(3) **Primers:** Normally the primer concentration is 50-100 pmol of each primer per reaction.
(4) **Template:** It needs experiences to estimate the amount of template added to a reaction.
(5) **$MgCl_2$:** $MgCl_2$ is variable in PCR, and it could be from 1 to 6 mM. It is important for the $MgCl_2$ amount added in PCR reaction.

4) The cycling reactions

There are three major steps in a PCR, which are repeated for 30 to 50 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

(1) **Denaturation** at 94°C: During the denaturation, the double strand melts open to single stranded DNA and all enzymatic reactions stop.
(2) **Annealing** at 54°C: The primers are moving around by Brownian motion and ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template.
(3) **Extension** at 72°C: The polymerase synthesizes DNA molecules.

The bases that are complementary to the DNA template are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side). Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene.

5) Before the PCR product is used in further applications, the following should be checked

(1) **There is a product formed:** It is possible that the quality of the DNA is poor, and one of the primers doesn't fit, or that there is too much starting template
(2) **The product is of the right size:** It is possible that there is a product, for example a band of 500 bp, but the expected gene should be 1800 bp long. It is also possible that both primers fit on a totally different gene.
(3) **Only one band is formed:** It is possible that the primers fit on the desired locations, and also on other locations. In that case, it can have different bands in one lane on a gel.

6) PCR buffers

A commonly used PCR buffer includes only KCl, Tris and $MgCl_2$ (for example, Perkin Elmer Cetus). A more complex buffer was previously proposed for multiplex reactions of the DMD gene exons (Chamberlain, 1988). The buffers are compared in multiplex PCR reactions, for their efficiency in supporting the activity of the Taq polymerase (Table 1).

Table 1. Comparison of PCR buffers

10× PCR buffer	5× DMD buffer
500 mM KCl 100 mM Tris-HCl (pH 8.3) 15 mM $MgCl_2$	83 mM $(NH4)_2SO_4$ 335 mM Tris-HCl (pH8.8) 33.5 mM $MgCl_2$ 50 mM ß-Mercapthoethanol 34 mM EDTA
Optimal dNTP concentration in the reaction=0.2 μM	Optimal dNTP concentration in the reaction=6 μM

7) Primers

A primer is a nucleic acid strand, or a related molecule that serves as a starting point for DNA replication. A primer is required because most DNA polymerases can only begin synthesizing a new DNA strand from an existing strand of nucleotides. In most natural DNA replication, the ultimate primer for DNA synthesis is a short strand of RNA. This RNA is produced by an RNA polymerase, and is later removed and replaced with DNA by a DNA polymerase.

Many laboratory techniques of molecular biology that involve DNA polymerases require primers. The primers are usually short artificially synthesized DNA molecules about 20 bp. The actual construction of such primers starts with 3'-hydroxyl nucleosides attached to a so-called controlled-pore glass. The 5'-hydroxyl of the nucleosides is covered dimethoxytrityl, which prevents the building of a nucleotide chain. To add a nucleotide, dimethoxytrityl is chemically removed, and the nucleotide is added. The 5'-hydroxyl of the new nucleotide is blocked by dimethoxytrityl, preventing the addition of more than one nucleotide to each chain. After that, the cycle is repeated for each nucleotide in the primer. DNA sequencing determines the nucleotides in a DNA strand. The sequencing method dideoxy sequencing, also known as chain termination method or Sanger method, uses a primer as a start marker for the chain reaction.

For PCR, the length of primers is usually not more than 50 nucleotides, and they match exactly the beginning and the end of the DNA fragment to be amplified. They anneal to the DNA template at these starting and ending points, where the DNA polymerase binds and begins the synthesis of the new DNA strand.

8) Primer design

Primer design is important for PCR. For the primer design, first it needs to get the target DNA sequence (it can be gotten from GenBank) and load the squence to computer to get primer sequence by the primer desgin software. Right now, there are many softwares for the primer design. The choice of the length of the primers and their melting temperature (T_m) depends on a number of considerations. The melting or annealing temperature of a primer is defined as the temperature below which the primer will anneal to the DNA template and above which the primer will dissociate from DNA template. Primers that are too short would anneal at several positions on a long DNA template, which would result in non-specific copies. T_m that is too high, i.e., above 80°C, can cause problems since the DNA-polymerase is less active at such temperatures. The optimum length of a primer is generally from 20 to 30 nucleotides with a T_m between 55°C and 65°C. There are several ways to calculate the primer T_m. As the following description, A, G, C and T are the number of that nucleotides in the primer, respectively, and $[Na^+]$ is the concentration of Na^+ in the PCR vial:

- "GC"-method: Fast and simple, for primers with more than 13 nucleotides:

$$T_M = 64 + \frac{G + C - 16.4}{A + G + C + T}$$

- "Salt-adjusted"-method: More accurate than GC, for primers with more than 13 nucleotides:

$$T_M = 100.5 + 41 * \frac{C + G}{A + C + G + T} - \frac{820}{A + C + G + T} * 16.6 * \log_{10}([\mathrm{Na}^+])$$

- Base-stacking calculation: Most accurate, but complicated:

$$T_M = \frac{\Delta H \frac{\mathrm{cal}}{\mathrm{mol}}}{\Delta S + Rln(\frac{primer}{2})} - 273.15\ ^\circ\mathrm{C}$$

where, ΔH is the enthalpy of base stacking interactions adjusted for helix initiation factors and ΔS is the entropy of base stacking adjusted for helix initiation factors and for the contributions of salts to the entropy.

R is the universal gas constant

$$\left(\frac{1.987\ \mathrm{cal}}{\mathrm{mol} \cdot ^\circ \mathrm{C}}\right)$$

A primer should not easily anneal with itself or others of its kind, building loops or hairpins in the process, even small hairpins are usually unavoidable.

Sometimes degenerate primers are used. These are actually mixtures of similar, but not identical, primers. They may be convenient if the same gene is to be amplified from different organisms, as the genes themselves are probably similar but not identical. When using the protein sequence as the primer design information, there are some problems. As several different codons can code for one amino acid, it is often difficult to deduce which codon is used in a particular case. The problem can be partly solved by using touchdown PCR.

9) PCR product yield

The above mentioned considerations make primer design a very exacting process, upon which product yield depends on:

(1) GC-content should be between 40-60%.
(2) Calculated T_m for both primers used in reaction should not differ >5°C and T_m of the amplification product should not differ from primers by >10°C.
(3) Annealing temperature usually is 5°C below the calculated lower T_m. However, it should be chosen empirically for individual conditions.
(4) Inner self-complementary hairpins of >4 and of dimers >8 should be avoided.
(5) Primer 3' terminus design is critical to PCR success since the primer extends from the 3' end. The 3' end should not be complementary over greater than 3-4 bp to any region of the other primer used in the reaction and must provide correct base matching to template.

RT-PCR primers sets may be designed using standard primer design algorithms without any modification. As with all PCR amplifications, however, the specific reaction conditions for each set must be optimized, particularly primer concentration, annealing temperature and magnesium chloride concentration.

10) Brief steps of traditional PCR

Brief steps of traditional PCR can be done as the following:

(1) The DNA strands are denatured at high temperature (around 95°C).
(2) The temperature is lowered and primers are added (around 52°C). The primers bond to their specific sites at this temperature.
(3) The temperature is brought back up to medium temperature and new DNA strands are synthesized by Taq polymerase (around 72°C).
(4) Repeat the above steps 1-3 for n cycles, amplifying the DNA (20-50 cycles).
(5) The product of PCR is 2^n copies of the selected DNA strand, where n is the number of cycles run.

The PCR process usually consists of a series of 20-50 cycles. Each cycle consists of three steps.

(1) The double-stranded DNA has to be heated to 94-96°C (or 98°C if extremely thermostable polymerases are used) in order to separate the strands. This step is called denaturing; it breaks apart the hydrogen bonds that connect the two DNA strands. Prior to the first cycle, the DNA is often denatured for an extended time to ensure that both the template DNA and the primers have completely separated and are now single-strand only. Time: usually 1-2 min, but can be to 5 min. Also certain polymerases are activated at this step.

(2) After separating the DNA strands, the temperature is lowered so the primers can attach themselves to the single DNA strands. This step is called annealing. The temperature of this stage depends on the primers and is usually 5°C below their T_m (45-60°C). A wrong temperature during the annealing step can result in primers not binding to the template DNA at all, or binding at random. Time is 1-2 min.

(3) Finally, the next step is for the DNA polymerase to copy the DNA strands. It starts at the annealed primer and works its way along the DNA strand. This step is called elongation. The elongation temperature depends on the DNA polymerase. This step takes 1 min per thousand base pairs. A final elongation step is frequently used after the last cycle to ensure that any remaining single stranded DNA is completely copied. This differs from all other elongation steps, only in that it is longer, typically 10-15 min. This last step is highly recomendable if the PCR product is to be ligated into a T vector using TA-cloning.

11) PCR examples

The floowing is given an example for the reagents used in a PCR program.

The reaction mixture:

(1) 1 µl DNA template (100 ng/µl)
(2) 2.5 µl primer (1.25 µl per primer, 100 ng/µl)
(3) 1 µl Pfu-polymerase
(4) 1 µl nucleotides
(5) 5.0 µl buffer solution
(6) 89.5 µl water

After the above reaction mixture is prepared, a 0.2 ml reaction tube containing the 0.1 ml of the above mixture is inserted into the thermocycler.

The PCR process consists of the following steps:

(1) **Initialization:** The mixture is heated at 94°C for 5 minutes to ensure that the DNA strands as well as the primers have melted. The DNA Polymerase can be present at initialization, or it can be added after this step.

(2) **Melting:** It is heated at 94°C for 30 seconds. For each cycle, this is usually enough time for the DNA to denature.

(3) **Annealing by heating at 55°C for 30 seconds:** The primers are jiggling around, caused by the Brownian motion. Short bondings are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer and on that little piece of double stranded DNA, the polymerase can attach and starts copying the template. Once there are a few bases built in, the T_m of the double-stranded region between the template and the primer is greater than the annealing or extension temperature.

(4) **Elongation by heating 72°C for 45 seconds:** This is the ideal working temperature for the polymerase. The primers, having been extended for a few bases, already have a stronger hydrogen bond to the template than the forces breaking these attractions. Primers that are on positions with no exact match, melt away from the tempplate and are not extended.

The bases are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template):

(1) Steps 2-4 are repeated 40 times, but with good primers and fresh polymerase, 20 to 30 cycles could be sufficient.

(2) Mixture is held at 72°C. This can run overnight. The DNA will not be damaged at 72°C after just one night.

The PCR product can be identified by its size using agarose gel electrophoresis. Agarose gel electrophoresis is a procedure that consists of injecting DNA into agarose gel and then applying an electric current to the gel. As a result, the smaller

DNA strands move faster than the larger strands through the gel toward the positive current. The size of the PCR product can be determined by comparing it with a DNA ladder, which contains DNA fragments of known size, also within the gel.

12) PCR optimization

Since PCR is very sensitive, it is important to avoid contamination from other DNA present in lab environment (bacteria, viruses, researchers' DNA). DNA sample preparation, reaction mixture assemblage and the PCR process, in addition to the subsequent reaction product analysis, should be performed in separate areas. For the preparation of reaction mixture, a laminar flow cabinet with UV lamp could be used. Fresh gloves should be used for each PCR step as well as displacement pipettes with aerosol filters, especially for the reverse transcription PCR using mRNA as the template. The reagents for PCR should be prepared separately and used solely for this purpose. Aliquots should be stored separately from other DNA samples. A control reaction (inner control), omitting template DNA, should always be performed, to confirm the absence of contamination or primer multimer formation.

13) Problems of PCR

PCR is not a perfect technique (nothing perfect in the world!), so that errors and mistakes can occur. These are some common errors and problems that may occur.

14) Polymerase errors

Taq polymerase lacks a 3' to 5' exonuclease activity. This makes it impossible for it to check the base it has inserted and remove it if it is incorrect, a process common in higher organisms. This in turn results in a high error rate of approximately 1 in 10,000 bases, which, if an error occurs early, can alter large proportions of the final product. Other polymerases are available for accuracy in vital uses such as amplification for sequencing. Examples of polymerases with 3' to 5' exonuclease activity include: KOD DNA polymerase, a recombinant form of *Thermococcus kodakaraensis* KOD1; Vent, which is extracted from *Thermococcus litoralis*; Pfu DNA polymerase, which is extracted from *Pyrococcus furiosus*; and Pwo, which is extracted from *Pyrococcus woesii*.

Pfu DNA polymerase is an enzyme found in the hyperthermophilic archaeon *Pyrococcus furiosus*, where it functions *in vivo* to replicate the organism's DNA. *In vitro*, Pfu is used to quickly amplify DNA in PCR, where the enzyme serves the central function of copying a new strand of DNA during each extension step. The main difference between Pfu and alternative enzymes is Pfu's superior thermostability and proofreading properties compared to other thermostable polymerases. Unlike Taq DNA polymerase, Pfu DNA polymerase possesses 3' to 5' exonuclease proofreading activity, meaning that it work its way along the DNA from the 3' end to the 5' end and correct nucleotide-misincorporation errors. This means that Pfu DNA polymerase-generated PCR fragments will have fewer errors than Taq-generated PCR inserts. As a result, Pfu is more commonly used for molecular cloning of PCR fragments than the historically popular Taq. Commercially available Pfu typically results in an error rate of 1 in 1.3 million base pairs and can yield 2.6% mutated products when amplifying 1 kb fragments using PCR. However, Pfu is slower and typically requires 1–2 min to amplify 1 kb of DNA at 72°C. Using Pfu DNA polymerase in PCR reactions also results in blunt-ended PCR products. Pfu DNA polymerase is superior for techniques that require high-fidelity DNA synthesis, but can also be used in conjunction with Taq polymerase to obtain the fidelity of Pfu with the speed of Taq polymerase activity.

15) Size limitations

PCR works readily with DNA of 1000-4000 bp, but above 5000 bp the polymerase tends to fall off and the typical heating cycle does not leave enough time for polymerisation to complete. It is possible to amplify larger pieces of up to 50,000 bp, with a slower heating cycle and special polymerases. These special polymerases are often polymerases fused to a DNA-binding protein, making them literally stick to the DNA longer.

16) Nonspecific binding of primer

The nonspecific binding of primers is always a possibility due to sequence duplications, nonspecific binding and partial primer binding, leaving the 5' end unattached. This is increased by the use of degenerate sequences or bases in the primer. Manipulation of annealing temperature and magnesium ion concentrations can increase specificity. Nonspecific priming can be prevented during the low temperatures of reaction preparation by use of hot-start polymerase enzymes where the active site is blocked by an antibody or chemical that only dislodges once the reaction is heated to 95˚C during the denaturation step of the first cycle. Other methods to increase specificity include Nested PCR and Touchdown PCR (Wikipedia, 2006).

17) Nested PCR Process

Nested PCR is a modification of PCR to reduce the contaminations in products due to the amplification of unexpected primer binding sites. One of the limitations of conventional PCR is that it requires primers complementary to the termini of the target DNA, and it is a common problems that the primers bind to incorrect regions of the DNA, giving unexpected products. Nested PCR involves two sets of primers, used in two successive runs of PCR, the second set intended to amplify a secondary target within the first run product.

(1) The target DNA undergoes the first run of PCR with the first set of primers, shown in green. The selection of alternative and similar primer binding sites gives a selection of products, only one containing the intended sequence.
(2) The product from the first reaction undergoes a second run with the second set of primers, shown in red. It is very unlikely that any of the unwanted PCR products contain binding sites for both the new primers, ensuring the product from the second PCR has little contamination from unwanted products of primer mis-binding and alternative primer target sequences.

Touchdown PCR or touchdown style PCR is a method of PCR by which degenerate primer will avoid amplifying nonspecific sequence. The temperature at which primers anneal during a PCR cycle determines the annealing. The primer melting point sets the upper limit on annealing temperature. At temperatures just below this point, only very specific base pairing between the primer and the template will occur. At lower temperatures, the primers bind less specifically.

The earliest steps of a touchdown PCR cycle have high annealing temperatures. For every subsequent 2 cycles, the annealing temperature is decreased by 1^{o}C. The primer will anneal at the highest temperature which is least-permissive of nonspecific that it is able to tolerate. The first sequence amplified is the one between the regions of greatest primer specificity. These fragments will be further amplified during subsequent rounds at lower temperatures, and will swamp out the nonspecific sequences to which the primers will bind at those lower temperatures. If the primer initially binds to the sequence of interest at a low temperature, subsequent rounds of PCR can be performed upon the product to further amplify those fragments.

18) Practical modifications to the PCR technique

(1) **Nested PCR**: Nested PCR is intended to reduce the contaminations in products due to the amplification of unexpected primer binding sites. Two sets of primers are used in two successive PCR runs, the second set intended to amplify a secondary target within the first run product.
(2) **Inverse PCR**: Inverse PCR is a method used to allow PCR when only one internal sequence is known. This is especially useful in identifying flanking sequences to various genomic inserts.
(3) **Reverse Transcription PCR**: Essentially normal PCR preceded by transcription by reverse transcriptase (to convert the RNA to cDNA) this is widely used in expression mapping, determining when and where certain genes are expressed.
(4) **Asymmetric PCR**: Asymmetric PCR is used to preferentially amplify one strand of the original DNA more than the other. It finds use in some types of sequencing and hybridization probing where having only one of the two complementary stands is ideal.
(5) **Quantitative PCR**: Quantitative PCR (qPCR) is used to rapidly measure the quantity of PCR product (real-time), thus is an indirect method for quantitatively measuring starting amounts of DNA, cDNA or RNA. This is commonly used for the purpose of determining whether a sequence is present or not, and if it is present the number of copies in the sample.
(6) **RT-PCR:** RT-PCR uses fluorescent dyes and probes to measure the amount of amplified product in real time, normally mRNA.
(7) **Touchdown PCR**: Touchdown PCR is a variant of PCR that reduces nonspecific primer annealing by more gradually lowering the annealing temperature between cycles. As higher temperatures give greater specificity for primer binding, primers anneal first as the temperature passes through the zone of greatest specificity.
(8) **Colony PCR**: Bacterial clones (E. coli) can be screened for the correct ligation products. Selected colonies are picked with a sterile toothpick from an agarose

plate and dabbed into the master mix or sterile water.

(9) **RACE-PCR**: Rapid amplification of cDNA ends.

(10) **Multiplex-PCR**: The use of multiple, unique primer sets within a single PCR reaction to produce amplicons of varying sizes specific to different DNA sequences. By targeting multiple genes at once, additional information may be illicited from a single test run that otherwise would require several times the reagents and technician time to perform.

19) Recent developments in PCR techniques

A more recent method which excludes a temperature cycle, but uses enzymes, is helicase-dependent amplification.

(1) **TAIL-PCR**: TAIL-PCR was developed in 1995. it is the thermal asymmetric interlaced PCR.

(2) Meta-PCR: Meta-PCR was developed by Andrew Wallace, and it allows to optimize amplification and direct sequence analysis of complex genes.

(3) **Genetic fingerprinting:** Genetic fingerprinting is a forensic technique used to identify a person by comparing his or her DNA with a given sample, such as blood from person can be genetically compared to blood from a another person to make sure if they have paternity relathionship, or a blood from crime scene can be genetically compared to blood from a suspect. The sample may contain only a tiny amount of DNA, obtained from a source such as blood, semen, saliva, hair, or other organic material. Theoretically, just a single strand is needed. First, one breaks the DNA sample into fragments, then amplifies them using PCR. The amplified fragments are then separated using gel electrophoresis. The overall layout of the DNA fragments is called a DNA fingerprint. Since there is a very small possibility that two individuals may have the same sequences, the technique is more effective at acquitting a suspect than proving the suspect guilty. This small possibility was exploited by defense lawyers in the controversial O. J. Simpson case. A match however usually remains a very strong indicator also in the question of guilt.

(4) **Paternity testing:** Although the resulting fingerprints are unique (except for identical twins), genetic relationships, for example, parent-child or siblings, can be determined from two or more genetic fingerprints, which can be used for paternity tests. A variation of this technique can also be used to determine evolutionary relationships between organisms.

(5) **Detection of hereditary diseases:** The detection of hereditary diseases in a given genome is a long and difficult process, which can be shortened significantly by using PCR. Each gene in question can easily be amplified through PCR by using the appropriate primers and then sequenced to detect mutations. Viral diseases, too, can be detected using PCR through amplification of the viral DNA.

(6) **Cloning genes:** Cloning a gene is the process of isolating a gene from one organism and then inserting it into another organism. PCR is often used to amplify the gene, which can then be inserted into a vector such as a plasmid. The DNA can then be transferred into an organism where the gene and its product can be studied more closely. Expressing a cloned gene can also be a way of mass-producing useful proteins, for example medicines or the enzymes in biological washing powders.

(7) **Mutagenesis:** Mutagenesis is a way of making changes to the sequence of nucleotides in the DNA. There are situations in which one is interested in mutated copies of a given DNA strand. Mutations can be introduced into copied DNA sequences in two fundamentally different ways in the PCR process. Site-directed mutagenesis introduces a mutation at a specific location on the DNA strand. Usually, the desired mutation is incorporated in the primers used for the PCR program. Random mutagenesis is based on the use of error-prone polymerases in the PCR process. In the case of random mutagenesis, the location and nature of the mutations cannot be controlled.

(8) **Analysis of ancient DNA:** Using PCR, it becomes possible to analyze DNA that is thousands of years old. PCR techniques have been successfully used on animals, such as a forty-thousand-year-old mammoth, and also on human DNA, in applications ranging from the analysis of

Egyptian mummies to the identification of a Russian Tsar.

(9) **Genotyping of specific mutations:** Through the use of allele-specific PCR, it can easily determine which allele of a mutation or polymorphism an individual has. The 3' end of the allele-specific primer is modified, to only anneal if it matches one of the alleles.

(10) **Comparison of gene expression:** Once RNA is isolated it can be reverse transcribed back into DNA (cDNA), at which point traditional PCR can be applied to amplify the gene (RT-PCR). In most cases if there is more starting material (mRNA) of a gene then during PCR more copies of the gene will be generated. By running samples of amplified cDNA from differently treated organisms it can get a general idea of which sample expressed more of the gene of interest.

20) PCR Equipments by Applied Biosystems Corporation

Applied Biosystems Corporation (AB) supplies several PCR instruments, called Thermal Cyclers.

(1) 60-Well Plate and 0.5 ml Tube GeneAmp® PCR System 9700

GeneAmp® PCR System 9700 is specifically designed for the amplification of nucleic acids. The GeneAmp® PCR System 9700 consists of a base module and one of many interchangeable sample block modules. The 0.5 ml GeneAmp® PCR System 9700 is designed for use with 0.5 ml reaction tubes for all of your large volume PCR applications (Table 2).

Table 2. Tube GeneAmp® PCR System 9700

Product Name	Part Number	Quantity/Package	Price (US$)
GeneAmp® PCR System 9700, Base Module	N8050200	1 unit	4995
0.5 ml GeneAmp® PCR System 9700	4310899	2 pieces	6995
GeneAmp® PCR System 9700, 0.5 ml Sample Block Module	4309131	1 unit	2000

(2) 96-Well Thermal Cyclers - 2720 Thermal Cycler

This personal-sized 96-well thermal cycler is the ideal instrument for both basic PCR and cycle sequencing applications using 0.2 ml reaction tubes or 96-well reaction plates (Table 3).

Table 3. 2720 Thermal Cycler

Product Name	Part Number	Quantity/Package	Price (US$)
Applied Biosystems 2720 Thermal Cycler	4359659	1 piece	4395

(3) 96-Well GeneAmp® PCR System 9700

GeneAmp® PCR System 9700 is specifically designed for the amplification of nucleic acids. The GeneAmp® PCR System 9700 consists of a base module and one of many interchangeable sample block modules. The 96-well GeneAmp® PCR System 9700 is designed for use with 0.2 ml reaction tubes or 96-well reaction plates for all of your routine PCR applications (Table 4).

Table 4. 96-Well GeneAmp® PCR System 9700

Product Name	Part Number	Quantity/Package	Price (US$)
Aluminum 96-Well GeneAmp® PCR System 9700	4314879	2 pieces	7095
Aluminum 96-Well GeneAmp® PCR System 9700, Sample Block Module	4314445	1 unit	2100
GeneAmp® PCR System 9700, Base Module	N8050200	1 unit	4995
Gold-plated 96-Well GeneAmp® PCR System 9700	4314878	2 pieces	7995
Gold-plated Silver 96-Well GeneAmp® PCR System 9700, Sample Block Module	4314443	1 unit	3000
Silver 96-Well GeneAmp® PCR System 9700	N8050001	2 pieces	7745
Silver 96-Well GeneAmp® PCR System 9700 Sample Block Module	N8050251	1 unit	2750

(4) Dual 96-Well GeneAmp® PCR System 9700

GeneAmp® PCR System 9700 is specifically designed for the amplification of nucleic acids. The GeneAmp® PCR System 9700 consists of a base module and one of many interchangeable sample block modules. The Auto-Lid Dual 96-well GeneAmp® PCR System 9700 is designed for use with 0.2 ml reaction tubes or 96-well reaction plates for high-throughput PCR and cycle sequencing applications (Table 5).

Table 5. Dual 96-Well GeneAmp® PCR System 9700

Product Name	Part Number	Quantity/Package	Price (US$)
Dual 96-Well Sample Block Module	4342718	1 piece	6995
GeneAmp® PCR System 9700, Base Module	N8050200	1 unit	4995
Dual 96-Well GeneAmp® PCR System 9700	4343176	2 pieces	11995

(5) 9800 Fast Thermal Cycler

The Applied Biosystems 9800 Fast Thermal Cycler is an integral component of the Applied Biosystems 9800 Fast PCR System, which reduces PCR reaction time from 2 hours to as little as 25 minutes (Table 6).

Table 6. 9800 Fast Thermal Cycler

Product Name	Part Number	Quantity/Package	Price (US$)
Applied Biosystems 9800 Fast Thermal Cycler with 96-well Aluminum Sample Block Module	4352604	2 pieces	8995

(6) 384-Well Block Thermal Cyclers - Auto-Lid Dual 384-Well GeneAmp® PCR System 9700

GeneAmp® PCR System 9700 is specifically designed for the amplification of nucleic acids. This system consists of a base module and one of many interchangeable sample block modules. The Auto-Lid Dual 384-well GeneAmp® PCR System 9700 is designed for use with 384-well reaction plates for high-throughput PCR and cycle sequencing applications (Table 7).

Table 7. Auto-Lid Dual 384-Well GeneAmp® PCR System 9700

Product Name	Part Number	Quantity/Package	Price (US$)
GeneAmp® PCR System 9700, Base Module	N8050200	1 unit	4995
Auto-Lid Dual 384-Well GeneAmp® PCR System 9700	4314487	2 pieces	14000
Auto-Lid Dual 384 GeneAmp® PCR System 9700, Sample Block Module	4312904	1 unit	9000

(7) Dual 384-Well GeneAmp® PCR System 9700

GeneAmp® PCR System 9700 is specifically designed for the amplification of nucleic acids. The GeneAmp® PCR System 9700 consists of a base module and one of many interchangeable sample block modules. The Auto-Lid Dual 384-well GeneAmp® PCR System 9700 is designed for use with 384-well reaction plates for high-throughput PCR and cycle sequencing applications (Table 8).

Table 8. Dual 384-Well GeneAmp® PCR System 9700

Product Name	Part Number	Quantity/Package	Price (US$)
GeneAmp® PCR System 9700, Base Module	N8050200	1 unit	4995
Dual 384-Well GeneAmp® PCR System 9700	N8050002	2 pieces	12000
GeneAmp® PCR System 9700, Dual 384-Well Sample Block Module	N8050400	1 unit	6995

(8) System 9700 Networking Software

Applied Biosystems System 9700 Networking Software is an innovative software package that allows researchers to communicate with up to 31 GeneAmp® PCR System 9700 instruments simultaneously from one PC (Table 9).

Table 9. System 9700 Networking Software

Product Name	Part Number	Quantity/Package	Price (US$)
System 9700 Networking Software	4332267	1 kit	1500
System 9700 Networking Software Expansion Kit	4332266	1 kit	50

21) PCR Equipments by Bio-Rad PCR Products

(1) MJ Mini Gradient Thermal Cycler

The MJ Mini 48-well gradient thermal cycler offers individual researchers the quality and features of advanced cyclers. Unlike other personal thermal cyclers, the MJ Mini cycler offers thermal gradient technology — allowing you to optimize reactions for maximum efficiency and accurate quantitation. And with the addition of a MiniOpticon two-color RT-PCR detector, the MJ Mini cycler is converted to the most compact real-time system available.

(2) MyCycler Personal Thermal Cycler

The 96-well MyCycler thermal cycler offers high quality at an economical price. The 12 cm (4.7") high-resolution display presents graphics and options for easy, fast programming.

(3) iCycler Thermal Cycler

The iCycler thermal cycler offers excellent thermal performance, fast ramping, intuitive programming, a choice of reaction modules with different assay formats, and optional upgrades for RT-PCR. Many user-friendly features, including help screens and reference lists, guide you through experiments. The high-resolution graphical interface simplifies file and protocol management and allows printing of a variety of reports.

(4) DNA Engine (PTC-200) Peltier Thermal Cycler

The single-bay DNA Engine cycler, the namesake of the family, was the first cycler to provide a high level of thermal performance with swappable blocks and a compact design. The DNA Engine cycler can be converted to a DNA Engine Opticon 2 system, dedicated to two-color RT-PCR, or it can be used with the swappable Chromo4 four-color RT-PCR detector.

(5) DNA Engine Dyad Peltier Thermal Cycler

The dual-bay DNA Engine Dyad thermal cycler features a high-density graphical interface and color display. Point-and-click navigation through the software enables rapid input of protocols. For laboratories expecting increased sample throughput, the DNA Engine Dyad can be expanded to a four-bay system with the addition of a Dyad Disciple thermal cycler. The DNA Engine Dyad/Dyad Disciple tandem can run up to eight independent protocols when equipped with dual blocks.

(6) Dyad Disciple Peltier Thermal Cycler

(7) DNA Engine Tetrad 2 Peltier Thermal Cycler

The four-bay DNA Engine Tetrad 2 thermal cycler is a redesigned and updated version of the original DNA Engine Tetrad chassis, which was instrumental to the Human Genome Project. It delivers the same precise thermal performance as the DNA Engine Dyad cycler and original Tetrad

thermal cyclers, allowing easy transport of protocols across platforms.

(8) PTC-100 Peltier Thermal Cycler

7. RT-PCR

1) Principle of RT-PCR Methodology

Currently, there are three techniques for RNA measurement: Reverse transcription PCR, Northern blot analysis and RNase protection assay. Reverse transcription PCR is the most sensitive technique for mRNA detection and quantitation. Compared to the other two techniques for quantifying mRNA levels (Northern blot analysis and RNase protection assay) reverse transcription PCR can be used to quantify mRNA levels from much smaller samples. Theoretically, this technique is sensitive enough to enable quantitation of RNA from a single cell, even it normally needs 10^6 cells.

RT-PCR principle is based on the properties of the PCR reaction kinetics. A quantification of the PCR products synthesized during the PCR is obtained at each cycle, and a threshold is defined from the PCR cycle number curves. The threshold cycle (C_T) corresponds to the intersection of the threshold and the PCR amplification curve. The threshold is chosen to intersect with all the PCR amplification curves during their exponential phases.

RT-PCR can detect sequence-specific PCR products as they accumulate in real-time during the PCR amplification process. As the PCR product is produced, RT-PCR can detect their accumulation and quantify the number of substrates exist in the initial PCR mixture before amplification start.

RT-PCR was developed from the PCR technique that measured the amplification of small DNA amount. For RT-PCR, mRNA or total RNA is isolated from a particular sample before producing a DNA copy of cDNA of each RNA molecule. The gene expression levels are then further amplified from the cDNA mixture together with a housekeeping gene (internal control). Housekeeping genes are those whose expression levels remain roughly constant in all samples, such as actin, hypoxanthine-guanine phosphoribosyltransferase (HGP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For the RT-PCR principle, more mRNA is in a sample, the earlier it will be detected during repeated cycles of amplification. RT-PCR machines can detect the amount of fluorescent DNA and thus the amplification progress. Amplification of cDNA is followed by a curve that is an initial flat-phase and followed by an exponential phase. As the experiment reagents are used up, DNA synthesis slows and the exponential curve flattens into a plateau.

Threshold is a level of normalized reporter signal that is usde for C_T determination in real-time assays. The level is set to be above the baseline but sufficiently low to be within the exponential growth region of an amplification curve. The cycle number at which the fluorescence signal associated with a particular amplicon accumulation crosses the thrshold is referred to as the C_T. C_T is threshold cycle, the cycle number at whitch the fluorescence generated within a reaction crosses the threshold line. C_T values are logarithmic and are used either directly or indirectly for the quantitative analyses.

Normally a housekeeping gene will not have the same C_T value over all samples analysed. Many softwares and spreadsheets have been produced to input C_T values and produce a numerical output showing gene expression levels compared between different cell samples. Such programs also allow statistical analysis of data, such as calculation of standard deviation and standard error.

Currently four different chemical principles of methodology are available for RT-PCR: (1) TaqMan® (Applied Biosystems, Foster City, CA, USA); (2) Molecular Beacons; (3) Scorpions®; (4) SYBR® Green (Molecular Probes). All the four methods do the detection of PCR products via the generation of a fluorescent signal. TaqMan probes, Molecular Beacons and Scorpions depend on Förster Resonance Energy Transfer (FRET) to generate the fluorescence signal through the coupling of a fluorogenic dye molecule (5' end) and a quencher moeity (3' end) to the same or different oligonucleotide substrates. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA (Dharmaraj, 2006). The old method for RT-PCR is **e**nd-point RT-PCR (relative RT-PCR, competitive RT-PCR and comparative RT-PCR). In spite of the rapid advances made in the area of RT-PCR detection chemistries and instrumentation, the end-point RT-PCR still remains a very commonly used technique for measuring changes in gene-expression in small sample numbers.

2) TaqMan Probes

TaqMan probes depend on the 5'- nuclease activity of the DNA polymerase used for PCR to hydrolyze an oligonucleotide that is hybridized to the target amplicon. TaqMan probes are oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher moeity coupled to the 3' end. These probes hybridize to an

internal region of a PCR product. In the unhybridized state (5' end with fluorogenic dye binds 3' end with quencher), the proximity of the fluor and the quench molecules prevents the detection of fluorescent signal from the probe. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5'- nuclease activity of the polymerase cleaves the probe. This decouples the fluorescent and quenching dyes, and FRET no longer occurs. So that fluorescence increases in each cycle and the fluorescence increasing has a linear relationship with the amount of probe cleavage. Well-designed TaqMan probes require very little optimization. In addition, they can be used for multiplex assays by designing each probe with a unique fluor/quench pair. However, TaqMan probes can be expensive to synthesize, with a separate probe needed for each mRNA target being analyzed (a primer costs about US$20, and a probes costs about US$250).

3) Molecular Beacons

Like TaqMan probes, Molecular Beacons also use FRET to detect and quantitate the synthesized PCR product through a fluor coupled to the 5' end and a quench attached to the 3' end of an oligonucleotide substrate. Unlike TaqMan probes, Molecular Beacons are designed to remain intact during the amplification reaction, and must rebind to target in every cycle for signal measurement. Molecular Beacons form a stem-loop structure when free in solution (a hairpin, 5' end with fluorogenic dye binds 3' end with quencher). Thus, the close proximity of the fluor and quench molecules prevents the probe from fluorescing. When a Molecular Beacon hybridizes to a target, the fluorescent dye and quencher are separated, and the fluorescent dye emits light upon irradiation. Like TaqMan, Molecular Beacons can be used for multiplex assays by using separated fluor/quench moieties on each probe. As with TaqMan probes, Molecular Beacons can be expensive to synthesize, with a separate probe required for each target.

4) Scorpions

With Scorpion probes, sequence-specific priming and PCR product detection is obtained using a single oligonucleotide. The Scorpion probe maintains a stem-loop configuration in the unhybridized state. The fluorophore is attached to the 5' end and is quenched by a moiety coupled to the 3' end. The 3' portion of the stem also contains sequence that is complementary to the extension product of the primer. This sequence is linked to the 5' end of a specific primer via a non-amplifiable monomer. After extension of the Scorpion primer, the specific probe sequence is able to bind to its complement within the extended amplicon thus opening up the hairpin loop and the fluorescence signal is observed.

5) SYBR Green

SYBR Green provides the simplest and most economical format for detecting and quantitating RT-PCR products. SYBR Green binds double-stranded DNA, and upon excitation emits light. Fluorescence increases as the PCR products accumulate. The advantages of SYBR Green are that it is inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other nonspecific reaction products, which results in an overestimation of the target concentration. For single PCR product reactions with well designed primers, SYBR Green can work extremely well and the spurious nonspecific background only shows up in very late cycles. SYBR Green is the most economical choice for RT-PCR product detection. Since the dye binds to double-stranded DNA, there is no need to design a probe for any particular target being analyzed. However, detection by SYBR Green requires extensive optimization. Since the dye cannot distinguish specific and nonspecific products accumulated in PCR, follow up assays are needed to validate results.

6) Real-time Reporters for Multiplex PCR

TaqMan probes, Molecular Beacons and Scorpions allow multiple DNA species to be measured in the same sample (multiplex PCR), since fluorescent dyes with different emission spectra may be attached to the different probes. Multiplex PCR allows internal controls to be co-amplified and permits allele discrimination in single-tube, homogeneous assays. These hybridization probes afford a level of discrimination impossible to obtain with SYBR Green, since they will only hybridize to true targets in a PCR and not to primer-dimers or other spurious products.

7) End-Point RT-PCR (Relative RT-PCR, Competitive RT-PCR and Comparative RT-PCR)

End-point RT-PCR can be used to measure changes in expression levels using three different methods: relative, competitive and comparative. The most commonly used procedures for quantitating end-point RT-PCR results rely on detecting a fluorescent dye such as ethidium bromide, or quantitation of P^{32}-labeled PCR

product by a phosphorimager or, to a lesser extent, by scintillation counting.

Relative quantitation compares transcript abundance across multiple samples, using a co-amplified internal control for sample normalization. Results are expressed as ratios of the gene-specific signal to the internal control signal. This yields a corrected relative value for the gene-specific product in each sample and these values may be compared between samples for an estimate of the relative expression of target RNA in the samples.

Using competitive RT-PCR, absolute quantitation measures the absolute amount of a specific mRNA sequence in a sample. Dilutions of a synthetic RNA are added to sample RNA replicates and are co-amplified with the endogenous target. The PCR product from the endogenous transcript is then compared to the concentration curve created by the synthetic competitor RNA.

Comparative RT-PCR mimics competitive RT-PCR in that target message from each RNA sample competes for amplification reagents within a single reaction, making the technique reliably quantitative. Because the cDNA from both samples have the same PCR primer binding site, one sample acts as a competitor for the other, making it unnecessary to synthesize a competitor RNA sequence.

Both relative and competitive RT-PCR quantitation techniques require pilot experiments. In relative RT-PCR, pilot experiments include selection of a quantitation method and determination of the exponential range of amplification for each mRNA under study. For competitive RT-PCR, a synthetic RNA competitor transcript must be synthesized and used in pilot experiments to determine the appropriate range for the standard curve. Comparative RT-PCR yields similar sensitivity as relative and competitive RT-PCR, but requires significantly less optimization and does not require synthesis of a competitor.

(1) Relative RT-PCR

Relative RT-PCR uses primers for an internal control that are multiplexed in the same RT-PCR reaction with the gene specific primers. Internal control and gene-specific primers must be compatible. The expression of the internal control should be constant across all samples being analyzed. Then the signal from the internal control can be used to normalize sample data to account for tube-to-tube differences caused by variable RNA quality or RT efficiency, inaccurate quantitation or pipetting. Common internal controls include GAPDH mRNAs, ß-actin and 18S rRNA. Unlike Northern blot and nuclease protection assays, where an internal control probe is simply added to the experiment, the use of internal controls in relative RT-PCR requires substantial optimization.

PCR reaction must be terminated when the products from both the internal control and the gene of interest are detectable and are being amplified within exponential phase. Because internal control RNAs are typically constitutively expressed housekeeping genes of high abundance, their amplification surpasses exponential phase with very few PCR cycles. It is therefore difficult to identify compatible exponential phase conditions where the PCR product from a rare message is detectable. Detecting a rare message while staying in exponential range with an abundant message can be achieved several ways: (A) by increasing the sensitivity of product detection; (B) by decreasing the amount of input template in RT or PCR reactions; (C) by decreasing the number of PCR cycles.

As an internal control 18S rRNA shows less variance in expression across treatment conditions than and ß-actin. However, as 18S rRNA is abundant in cells, it is difficult to detect the PCR product for rare messages in the exponential phase of amplification of 18S rRNA.

The biochemical company Ambion's patented Competimer™ Technology solves this problem by attenuating the 18S rRNA signal even to the level of rare messages. Attenuation is made from the use of competimers that are the primers identical in sequence to the functional 18S rRNA primers but that are blocked at their 3' end and cannot be extended by PCR. Competimers and primers are mixed at various ratios to reduce the amount of PCR product generated from 18S rRNA. Ambion's QuantumRNA 18S Internal Standards contain 18S rRNA primers and competimers designed to amplify 18S rRNA in all eukaryotes. The Universal 18S Internal Standards function across the broadest range of organisms including plants, animals and many protozoa. The Classic I and Classic II 18S Internal Standards by Ambion can be used with any vertebrate RNA sample. All 18S Internal Standards work well in multiplex RT-PCR.

(2) Competitive RT-PCR

Competitive RT-PCR precisely quantitates a message by comparing RT-PCR product signal intensity to a concentration curve generated by a synthetic competitor RNA sequence. The competitor RNA transcript is designed for amplification by the same primers and with the same efficiency as the endogenous target. The competitor produces a different-sized product so that it can be distinguished from the endogenous target product by gel analysis. The competitor is

carefully quantitated and titrated into replicate RNA samples. Pilot experiments are used to find the range of competitor concentration where the experimental signal is most similar. Finally, the mass of product in the experimental samples is compared to the curve to determine the amount of a specific RNA present in the sample. Some protocols use DNA competitors or random sequences for competitive RT-PCR.

(3) Comparative RT-PCR

Relative RT-PCR requires extensive optimization to ensure that the PCR is terminated when both the gene of interest and an internal control are in the exponential phase of amplification. Competitive RT-PCR requires that an exogenous competitor be synthesized for each target to be analyzed. However, comparative RT-PCR achieves the same level of sensitivity as these standard methods of qRT-PCR with significantly less optimization. Target mRNAs from 2 samples are assayed simultaneously, each serving as a competitor for the other, making it possible to compare the relative abundance of target between samples. Comparative RT-PCR is ideal for analyzing target genes discovered by screening methods such as array analysis and differential display.

8) Brief Description for the RT-PCR Procedure (Protocol online, 2006)

(1) The first step of the RT-PCR is to isolate RNA and then do the reverse transcription PCR, and TRIzol reagent can be used (Invitrogen Corporation**,** California, USA) for the isolation of RNA. Isolated RNA could be dissolved in diethyl-pyrocarbonate (DEPC) treated water, and the isolated RNA samples can be stored at -70^{o}C until used. The same biological samples used for RNA isolation with TRIzol are also can be saved to isolate protein and DNA. The expected yield of RNA from 1 x 10^{6} cultured cells is: epithelial cells, 8-15 µg fibroblasts, 5-7 µg.
(2) mRNA or total RNA is used as template for the cDNA synthesis by reverse transcriptase using an oligo dT primer. In RT-PCR, it usually uses a reverse transcriptase that has an endo H activity. This removes the mRNA allowing the second strand of DNA to be formed. A PCR mix is then set up which includes a heat-stable polymerase (such as Taq polymerase), specific primers for the gene of interest, deoxynucleotides and a suitable buffer.
(3) cDNA is denatured at around 90-96^{o}C. The sample is cooled to 50^{o}C to 60^{o}C and specific primers are annealed that are complementary to a site on each strand. The primers sites may be up to 600 bases apart but are often about 100 bases apart.
(4) The temperature is raised to 72^{o}C and the heat-stable Taq DNA polymerase extends the DNA from the primers. The four cDNA strands (two old and two new strands) are denatured again at approximately 94^{o}C.
(5) The primers are annealed at a suitable temperature (normally between 50^{o}C and 60^{o}C) again.
(6) Change to 72^{o}C and new DNA strands are synthesized by Taq DNA polymerase again, and 8 DNA strands present now.
(7) Again, the strands are denatured by raising the temperature to 94^{o}C and then the primers are annealed at 60^{o}C.
(8) The temperature is raised and the Taq polymerase copies the eight strands to sixteen strands.
(9) The strands are denatured and primers are annealed.
(10) The fourth cycle results in 32 strands.
(11) Another round doubles the number of single stands to 64.
(12) The rounds continue to 30-50 cycles and it will get 2^{30-50} DNA strands.

After 30 to 50 rounds of synthesis of cDNA, the reaction products can be analyzed by agarose gel electrophoresis. The gel is stained with ethidium bromide. This type of agarose gel-based analysis of cDNA products of reverse transcriptase-PCR does not allow accurate quantitation since ethidium bromide is rather insensitive and when a band is detectable, the logarithmic stage of amplification is over. Ethidium bromide is a dye that binds to double stranded DNA by interpolation between the base pairs. Here it fluoresces when irradiated in the UV part of the spectrum. However, the fluorescence is not very bright. Other dyes such as SYBR green and TaqMan Gene Expression Assays that are much more fluorescent than ethidium bromide are used in RT-PCR now.

SYBR green is a dye that binds to double stranded DNA but not to single-stranded DNA and is frequently used in RT-PCR reactions. When it is bound to double stranded DNA it fluoresces more brightly than ethidium bromide. Other methods

such as TaqMan Gene Expression Assays can be used to detect the product during RT-PCR.

A gene that is to be used as a loading control (or internal standard) should have various features:

(1) The standard gene should have the same copy number in all cells
(2) It should be expressed in all cells
(3) A medium copy number is advantageous since the correction should be more accurate

Commonly used standards are:

(1) GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) mRNA
(2) ß-actin mRNA
(3) MHC I (major histocompatibility complex I) mRNA
(4) Cyclophilin mRNA
(5) mRNAs for certain ribosomal proteins e.g. RPLP0 (ribosomal protein, large, P0). This is also known as 36B4, P0, L10E, RPPO, PRLP0, 60S acidic ribosomal protein P0, ribosomal protein L10, Arbp or acidic ribosomal phosphoprotein P0.
(6) 28S or 18S rRNAs (ribosomal RNAs)

Normally the sample preparation (cell/tissue obtained and RNA isolation, etc) is the most time cost for the research and the time cost is different for the different experiments. The following table gives the minimum time required for RT-PCR experiment (Table 10).

Table 10. Time required for RT-PCR

Steps	Running	Time (hours)
1	cDNA synthesis	2
2	RT-PCR	2
3	Dissociation curve analysis	0.5
Sum	Total Time	4.5

9) Quantitation of RT-PCR Results

Normally, either of the two methods can be used to quantify RT-PCR results: the standard curve method and the comparative threshold method.

(1) Standard Curve Method

In standard curve method, a standard curve is constructed from an RNA of known concentration. This curve is then used as a reference standard for extrapolating quantitative information for target mRNA. Though RNA standards can be used, their stability can be a source of variability in the final analyses. In addition, using RNA standards would involve the construction of cDNA plasmids that have to be in vitro transcribed into the RNA standards and accurately quantitated, a time-consuming process. However, the use of absolutely quantitated RNA standards will help generate absolute copy number data.

In addition to RNA, other nucleic acid samples can be used to construct the standard curve, including purified plasmid dsDNA, in vitro generated ssDNA or any cDNA sample expressing the target gene. OD_{260nm} can be used to assess the concentration of these DNAs, which can be converted to a copy number value based on the molecular weight of the sample used. cDNA plasmids are the preferred standards for standard curve quantitation. However, since cDNA plasmids will not control for variations in the efficiency of the reverse transcription step, this method will only yield information on relative changes in mRNA expression, and this can be corrected by normalization to a housekeeping gene.

(2) Comparative C_T Method

C_T is the threshold cycle. The comparative C_T method involves comparing the C_T values of the samples with a control such as a non-treated sample or RNA from normal tissue. The comparative C_T values of both the control and the samples are normalized to an appropriate endogenous housekeeping gene.

Comparative C_T method is also known as the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_T = \Delta C_{T\ sample} - \Delta C_{T\ reference}$. $\Delta C_{T\ sample}$ is the C_T value for any sample normalized to the endogenous housekeeping gene and $\Delta C_{T\ reference}$ is the C_T value for the control normalized to the endogenous housekeeping gene.

For the $\Delta\Delta C_T$ calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be established by looking at how ΔC_T varies with template dilution. If the plot of cDNA dilution versus ΔC_T is close to zero, it implies that the efficiences of the target and housekeeping genes are similar.

10) RT-PCR Equipments by Applied Biosystem Corporation

RT-PCR requires an instrumentation platform that consists of a thermal cycler, a computer, optics for fluorescence excitation and emission collection, and software for data acquisition and analysis. These machines, available from several manufacturers, differ in sample capacity (some are

96-well standard format, some process fewer samples or require specialized glass capillary tubes), method of excitation (some use lasers, some broad spectrum light sources with tunable filters), and overall sensitivity. There are also platform-specific differences in how the software processes data. An RT-PCR machine cost about US$20,000 to US$150,000.

Now there are many RT-PCR equipments available and the parts do not match each other. As an example, the company of Applied Biosystems (Foster City, California, USA) has the following RT-PCR instruments:

(1) 96-Well and 0.2 ml Tube Instruments

A. 7000 RT-PCR System: The Applied Biosystems ABI Prism® 7000 Sequence Detection System will be effective up to March 31, 2006. After this date, the ABI Prism® 7000 Sequence Detection System will no longer be available for sale. The company decided to discontinue the sale of this product because of the introduction of newer and more affordable technology.

B. 7300 RT-PCR System: The Applied Biosystems 7300 RT-PCR System is an integrated platform for the detection and quantification of nucleic acid sequences. Applied Biosystems supplies a Dell™ Notebook with the 7300 System. The current price is US$34,900.

(2) 7500 RT-PCR System

The Applied Biosystems 7500 RT-PCR System is a leading edge system with features to support labs requiring more capabilities from their RT-PCR platform. The current price is US$42,500.

(3) 96-Well Instrument - 7500 Fast RT-PCR System

The Applied Biosystems 7500 Fast RT-PCR System enables high speed thermal cycling in a 96-well format, reducing the run times to less than 40 minutes and accelerating your research by providing access to results more quickly than ever before. The current price is US$49,900.

(4) 96- and 384-Well Instrument - 7900HT Fast RT-PCR System

The Applied Biosystems 7900HT Fast RT-PCR System is the only qRT-PCR system that combines 96- and 384-well plate compatibility and the TaqMan® Low Density Array with fully automated robotic loading and now also offers optional Fast RT-PCR capability. The current price is US$131,500.

Summarily, current prices of RT-PCR instruments by Applied Biosystems are described in the following (Table 11).

Table 11. Current prices of RT-PCR instruments by Applied Biosystems

Instruments	Price (US$)
The Applied Biosystems 7300 RT-PCR System	34,900
The Applied Biosystems 7500 RT-PCR System	42,500
The Applied Biosystems 7500 Fast RT-PCR System	49,900
7900HT Fast RT-PCR System	131,500

Applied Biosystems can be contacted:
850 Lincoln Centre Drive
Foster City, CA 94404, USA
Telephones: 650-638-5800, 1800-327-3002
Fax: 650-638-5884,
Email: NA_ActSrvInfo@appliedbiosystems.com
Website: http://www.appliedbiosystems.com)

11) RT-PCR Equipments by Bio-Rad

Described by Bio-Rad Company, each of Bio-Rad's RT-PCR detection systems is an upgrade that adds RT-PCR capabilities to a base thermal cycler. The detection systems incorporate optical modules that enable detection of target sequences labeled with fluorescent probes. Sample quantitation by RT-PCR increases reliability of gene expression analyses, improves viral load assessment, and accelerates throughput in screening applications. Bio-Rad's RT-PCR detection systems range from economical upgrades that allow two-target detection to sophisticated five-target detection systems (Bio-Rad, 2006).

(1) MiniOpticon RT-PCR Detection System

The MiniOpticon RT-PCR detection system comprises a compact two-color RT-detector built on the MJ Mini cycler. It is the smallest and most portable system available for RT-PCR applications and gel-free PCR analysis. Its two-color multiplexing capability allows detection of a range of fluorophores.

The MiniOpticon system uses illumination technology similar to that of the DNA Engine Opticon 2 system, with which samples are sequentially illuminated by a fixed array of 48 blue-green light-emitting diodes (LEDs). Each LED beam is precisely focused onto its corresponding well to ensure minimal cross talk and light scattering. Cross talk is further reduced by

sequentially illuminating and detecting each well. LEDs provide a long-lived, maintenance-free excitation source. Emitted fluorescence is detected by one of two sensitive, filtered photodiodes. This innovative no-moving-parts design allows accurate detection in a compact and robust package.

(2) MyiQ Single-Color RT-PCR Detection System

The MyiQ RT-PCR detection system offers an affordable alternative for the detection of common green fluorescent dyes such as FAM and SYBR Green I. This system interfaces directly with the iCycler thermal cycler, which offers superior features such as a thermal gradient and Peltier-effect driven thermal performance. The MyiQ RT-PCR detection system is designed for those just getting started with this technology, as well as those looking for additional instruments to handle increasing routine assay demands. The MyiQ system's performance specifications are similar to those of the iQ5.

(3) DNA Engine Opticon 2 RT-PCR Detection System

The DNA Engine Opticon 2 system has two-color detection capability and a sensitive optical system with no moving parts. Samples are illuminated by a fixed array of 96 blue-green LEDs and detected by two photomultiplier tubes (PMTs). The first PMT detects at 523-543 nm (suitable for detecting SYBR Green I and FAM-labeled probes), while the second detects at 540-700 nm (suitable for detecting many commonly used fluorophores, including HEX-, TET-, TAMRA-, and VIC dye-labeled probes).

The DNA Engine Opticon 2 can be used for singleplex or multiplex reactions. Multiplexing combines two or more reactions in a single tube, which saves time and reagents. Furthermore, multiplexing allows you to include internal controls to improve accuracy in many applications, and simplifies genotyping by allowing the detection of multiple alleles within the same tube. For full use of the power of multiplexing, Opticon Monitor software permits simultaneous viewing of data from two channels, allows plotting the fluorescence output of one dye against that of another for automated scoring of genotypes, and includes a function for calculating relative gene expression.

(4) Chromo4 RT-PCR Detection System

The Chromo4 detector brings real-time four-color multiplex quantitative PCR capabilities to the DNA Engine platform. The Chromo4 detector incorporates a 96-well Alpha unit, which allows the Chromo4 detector to be swapped with any Alpha unit on any DNA Engine chassis in seconds, with no tools required. The flexibility of the system is maintained, so other Alpha units can be remounted on the chassis for use in other applications.

(5) iQ5 RT-PCR Detection System

The iQ5 RT-PCR detection system is a modular upgrade to the iCycler thermal cycler with 96-well reaction module. With the iQ5 system, you can accurately quantitate targets over a dynamic range of more than 9 orders of magnitude. You can also perform qualitative assays for allelic discrimination or screening for known mutations. The iQ5 system offers five-target analysis capabilities for multiplex PCR and data collection and analysis options for gene expression analysis. The iQ5 system is compatible with multiple detection chemistries, providing maximum flexibility in RT-PCR applications.

12) Reagents and Tools for RT-PCR

Table 12 gives the reagents and equipments needed for RT-PCR of SYBR method (Table 12).

Table 12. Reagents and equipments needed for RT-PCR (SYBR method)

No.	Reagents/Equipments for RT-PCR SYBR method
1	Oligonucleotide Primers.
2	Mouse total liver RNA (Stratagene).
3	Mouse total RNA master panel (BD Biosciences/Clontech).
4	SYBR Green PCR master mix, 200 reactions (Applied Biosystems).
5	Optical tube and cap strips (Applied Biosystems).
6	SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen).
7	25 bp DNA ladder (Invitrogen).
8	ABI Prism 7000 Sequence Detection System (Applied Biosystems).
9	ABI Prism 7000 SDS software (Applied Biosystems).
10	3% ReadyAgarose Precast Gel (Bio-Rad).
11	Agarose gel electrophoresis apparatus (Bio-Rad).

Table 13 gives the basic reagents and equipments needed for RT-PCR of TaqMan method (Table 13).

Table 13. Reagents and equipments needed for RT-PCR (TaqMan method)

No.	Reagents/Equipments for RT-PCR TaqMan method
1	Isolated RNA (RTIzol can be used for the RNA isolation)
2	Primer 1 (sense primer)
3	Primer 2 (antisense primer)
4	Probe with dye
5	RT-PCR enzymatic kit (one step or two step)
6	Reaction plates
7	RT-PCT instrument (e.g. ABI Prism 7000 Sequence Detection System, Applied Biosystems)

Ambion's MessageSensor™ RT Kit includes an RNase H+ MMLV RT that clearly outperforms MMLV RT enzymes that have abolished RNase H activity in RT-PCR experiments. Unlike many other qRT-PCR kits, MessageSensor includes a total RNA control, a control human GAPDH primer set, RNase inhibitor, and nucleotides, as well as a buffer additive that enables detection with SYBR® Green dye.

The Cells-to-cDNA™ II Kit produces cDNA from cultured mammalian cells in less than 2 hours. No RNA isolation is required. This kit is ideal for those who want to perform reverse transcription reactions on small numbers of cells, numerous cell samples, or for people who are unfamiliar with RNA isolation. Ambion's Cells-to-cDNA II Kit contains a novel Cell Lysis Buffer that inactivates endogenous RNases without compromising downstream enzymatic reactions. After inactivation of RNases, the cell lysate can be directly added to a cDNA synthesis reaction.

Genomic DNA contamination can lead to false positive RT-PCR results. Ambion offers a variety of tools for eliminating genomic DNA contamination from RNA samples prior to RT-PCR. Ambion's DNA-free™ DNase Treatment and Removal Reagents are designed for removing contaminating DNA from RNA samples and for the removal of DNase after treatment without Proteinase K treatment and organic extraction. In addition, Ambion has also developed TURBO™ DNase, a hyperactive enzyme engineered from wild-type bovine DNase. The proficiency of TURBO DNase in binding very low concentrations of DNA means that the enzyme is particularly effective in removing trace quantities of DNA contamination.

Ambion now also offers an economical alternative to the high cost of PCR reagents for the ABI 7700 and other 0.2 ml tube-based real-time instruments. SuperTaq™ Real-Time performs as well as the more expensive alternatives, and includes dNTPs and a Reaction Buffer optimized for SYBR Green, TaqMan, and Molecular.

13) Detailed Procedure of RT-PCR

RT-PCR is to quantify the gene expression in a real time. So that, the first step of the RT-PCR is to isolate RNA and then do the reverse transcription PCR. There are many methods to isolate RNA, and with the TRIzol reagent (Invitrogen Corporation**,** 1600 Faraday Avenue, PO Box 6482, Carlsbad, California 92008, USA; Phone: 760-603-7200) it is an easy way. Isolated RNA could be dissolved in diethyl-pyrocarbonate (DEPC) (0.01%) treated water that inactivates RNase. RNA concentration can be determined spectrophotometrically and a ratio of optical density $OD_{260\ nm}/OD_{280\ nm}>1.6$ can be considered as pure. TRIzol Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi. During sample homogenization or lysis, TRIzol Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. After removal of the aqueous phase, the DNA and proteins in the sample can be recovered by sequential precipitation.

Precipitation with ethanol yields DNA from the interphase, and an additional precipitation with isopropyl alcohol yields proteins from the organic phase. Copurification of the DNA may be useful for normalizing RNA yields from sample to sample. This technique performs well with small quantities of tissue (50-100 mg) and cells (5×10^6), and large quantities of tissue (>/=1 gram) and cells ($>10^7$), of human, animal, plant, or bacterial origin. The simplicity of the TRIzol Reagent method allows simultaneous processing of a large number of samples. The entire procedure can be completed in

one hour. Total RNA isolated by TRIzol Reagent is free of protein and DNA contamination. It can be used for Northern blot analysis, dot blot hybridization, poly(A)+ selection, in vitro translation, RNase protection assay, and molecular cloning.

For use in PCR, treatment of the isolated RNA with amplification grade DNase I (Invitrogen Cat. No. 18068-015) is recommended when the two primers lie within a single exon. TRIzol Reagent facilitates isolation of a variety of RNA species of large or small molecular size. For example, RNA isolated from rat liver, electrophoresed on an agarose gel, and stained with ethidium bromide, shows discrete bands of high molecular weight RNA between 7 Kb and 15 Kb in size, two predominant ribosomal RNA bands at approximately 5 Kb (28S) and at approximately 2 Kb (18S), and low molecular weight RNA between 0.1 and 0.3 Kb (tRNA, 5S). The isolated RNA has an A 260/280 ratio of 1.6-1.8. The expected yield of RNA per mg of tissue is: liver and spleen, 6-10 μg; kidney, 3-4 μg; skeletal muscles and brain, 1-1.5 μg; placenta, 1-4 μg.

In an RT-PCR reaction, a fluorescent reporter molecule is used to monitor the PCR as it progresses. The fluorescence emitted by the reporter molecule manifolds as the PCR product accumulates with each cycle of amplification. Based on the molecule used for the detection, RT-PCR techniques can be categorically placed under two heads: Nonspecific detection using DNA binding dyes and specific detection target specific probes (PREMIER Biosoft International, 2006).

(1) TaqMan primer and probe design

A. The Primer Express software designs primers with a T_m of 58-60^{0}C, and probes with a T_m value of 10^{o}C higher. The T_m of both primers should be equal.
B. Primers should be 15-30 bp.
C. The G+C content should be 30-80%. If a higher G+C content is unavoidable, the use of high annealing and T_m, cosolvents such as glycerol, DMSO, or 7-deaza-dGTP may be necessary.
D. The run of an identical nucleotide should be avoided, especially that Gs should not be more than 4.
E. The total number of Gs and Cs in the last five nucleotides at the 3' end of the primer should not exceed 2, and this helps to introduce relative instability to the 3' end of primers to reduce nonspecific priming.
F. Maximum amplicon size should not exceed 400 bp (prefered 50-150 bp). Smaller amplicons give more consistent results because PCR is more efficient and more tolerant of reaction conditions.
G. The probes should not have runs of identical nucleotides, G+C content should be 30-80%, there should be more Cs than Gs, and not a G at the 5' end. The higher number of Cs produces a higher ΔRn. The choice of probe should be made first.
H. To avoid false-positive results due to amplification of contaminating genomic DNA in the cDNA preparation, it is preferable to have primers spanning exon-exon junctions in the cDNA sequence. This way, genomic DNA will not be amplified.
I. If a TaqMan probe is designed for allelic discrimination, the mismatching nucleotide should be in the middle of the probe rather than at the ends.
J. Use primers that contain dA nucleotides near the 3' ends so that any primer-dimer generated is efficiently degraded by AmpErase UNG. If primers cannot be selected with dA nucleotides near the ends, the use of primers with 3' terminal dU-nucleotides should be considered.

The TaqMan probes from ABI normally are at 0.1 mM. If a 1/20 dilution is made, this gives a 5 μM solution. This stock solution should be aliquoted, frozen (-20^{o}C) and kept in the dark. Using 1 μL of this in a 50 μL reaction gives the recommended 100 nM final concentration.

The primers arrive lyophilised with the amount given on the tube in pmols (such as 150,000 pmol). If 1 nmol of primer is resuspended in 1 μL of H_2O, the resulting solution is 1 mM. It is best to freeze this stock solution in aliquots. When the 1 mM stock solution is diluted 1/100, the resulting working solution will be 10 μM. To get the recommended 50-900 nM final primer concentration in 50 μL reaction volume, 0.25-4.50 μL should be used per reaction (2.5 μL for 500 nM final concentration).

(2) Nonspecific detection using DNA binding dyes

In RT-PCR, DNA binding dyes are used as fluorescent reporters to monitor the PCR reaction. The fluorescence of the reporter dye increases as the product accumulates with each successive cycle of amplification. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase.

If a graph is drawn between the log of the starting amount of template and the corresponding increase the fluorescence of the reporter dye fluorescence during RT-PCR, a linear relationship is observed.

SYBR® Green is the most widely used double-strand DNA-specific dye reported for RT-PCR. SYBR® Green binds to the minor groove of the DNA double helix. In the solution, the unbound dye exhibits very little fluorescence. This fluorescence is substantially enhanced when the dye is bound to double stranded DNA. SYBR® Green remains stable under PCR conditions and the optical filter of the thermocycler can be affixed to harmonize the excitation and emission wavelengths. Ethidium bromide can also be used for detection but its carcinogenic nature renders its use restrictive.

Although these double-stranded DNA-binding dyes provide the simplest and cheapest option for RT-PCR, the principal drawback to intercalation based detection of PCR product accumulation is that both specific and nonspecific products generate signal.

(3) Specific detection using Target specific probes

Specific detection of RT-PCR is done with some oligonucleotide probes labeled with both a reporter fluorescent dye and a quencher dye. Probes based on different chemistries are available for real-time detection, these include:

A. Molecular Beacons
B. TaqMan® Probes
C. FRET Hybridization Probes
D. Scorpion® Primers

(4) Reverse Transcription Procedure

Reverse transcription is the process by which RNA is used as a template to synthesize cDNA. Among the first options to consider when selecting a method to perform the reverse transcription is whether to use a one-step real-time or a two-step real-time method. Now, many companies are offering reverse transcription reagent kit. The kit is classified into two types: one-step and two-step. As an example, Applied Biosystems offers the one-step reagent and two-step reagent of reverse transcription reagent kit.

A. **One-step reagent:** Requires single reaction mix because real-time and PCR occur in the same tube. It may get better limit of detection with rare transcripts and requires sequence-specific primer for cDNA synthesis. 2 µl of RNA solution was analyzed with a random reverse-transcriptase RT–PCR assay.
   a. The Superscript II platinum Taq polymerase one-step RT-PCR kit by Invitrogen contains 10 µl of buffer concentrate, 2 mM of magnesium sulphate, 0.8 µl of enzyme mixture and 1.9 µM of each of two primers (20 µl total volume).
   b. TaqMan® One-Step RT-PCR Master Mix Reagents of Applied Biosystems (AB) is another easy-to-use kit that contains all components needed for one-step RT-PCR applications and the kit employs AmpliTaq Gold® DNA polymerase for enhanced performance. It contains two components. **Vial 1:** AmpliTaq Gold® DNA Polymerase mix (2×) is optimized for 5 nuclease assays using TaqMan® probes and contains AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference 1, and optimized buffer components. **Vial 2:** RT enzyme mix (40×) contains MultiScribe™ Reverse Transcriptase and RNase Inhibitor. It costs US$590 for the 200 reactions and US$5420 for 2000 reactions as the current price (Table 14).
   c. TaqMan® EZ RT-PCR Core Reagents combine rT*th* DNA polymerase with the fluorogenic 5' nuclease assay in a one-step reverse transcription PCR format. This format is ideally suited to high sample throughput and provides the additional benefit of high temperature reverse transcription, which can prove beneficial when amplifying targets in regions of RNA with abundant secondary structure (Table 15).
   d. The TaqMan® Gold RT-PCR kit provides the individual components needed to perform reverse transcription PCR of RNA to cDNA using the 5' nuclease assay. This kit can be used to perform both one-step and two-step RT-PCR (Table 16).

Table 14. TaqMan® One-Step RT-PCR Master Mix Reagents of Applied Biosystems

Product Name	Part Number	Quantity/Package	Price (US$)
TaqMan® One-Step RT-PCR Master Mix Reagents Kit	4309169	200 reactions	590
10-Pack, TaqMan® One-Step RT-PCR Master Mix Reagents Kit	4313803	2000 reactions	5420

Table 15. TaqMan® EZ RT-PCR Core Reagents of Applied Biosystems

Product Name	Part Number	Quantity/Package	Price (US$)
TaqMan® EZ RT-PCR Core Reagents without Controls	N8080236	200 reactions	755
10-Pack, TaqMan® EZ RT-PCR Core Reagents	403028	2000 reactions	6900

Table 16. TaqMan® Gold RT-PCR kit of Applied Biosystems

Product Name	Part Number	Quantity/Package	Price (US$)
TaqMan® Gold RT-PCR Reagents without Controls	N8080232	200 reactions	755
10-Pack, TaqMan® Gold RT-PCR Reagents without controls	4304133	2000 reactions	6900

B. **Two-step reagent:** Requires two reaction mixes (real-time reaction and PCR reaction) and cDNA can be stored for later use. By using random primer, it can simultaneously reverse transcribe all mRNA as well as 18s rRNA (targets+endogenous controls), and it can use sequence-specific primer, random primer or oligo $d(T)_{16}$ for cDNA synthesis.

Reverse transcription is carried out with the SuperScript First-Strand Synthesis System for RT-PCR. The following procedure is based on Invitrogen's protocol. In addition, Bio-Rad and other companies also have complete kit for RT-PCR.

A. Prepare RNA/primer mixture in each tube (Table 17).

B. Incubate the samples at 65°C for 5 min and then on ice for at least 1 min.

C. Prepare reaction master mixture for each reaction (Table 18)

D. Add the reaction mixture to the RNA/primer mixture, mix briefly, and then place at room temperature for 2 min.

E. Add 1 μl (50 units) of SuperScript II RT to each tube, mix and incubate at 25°C for 10 min.

F. Incubate the tubes at 42°C for 50 min, heat inactivate at 70°C for 15 min, and then chill on ice.

G. Add 1 μl RNase H and incubate at 37°C for 20 min.

H. Store the first strand cDNA at -20°C until use for RT-PCR.

Table 17. RNA/primer mixture

Contents	Amount
Total RNA	5 μg
Random hexamers (50 ng/μl)	3 μl
10 mM dNTP mix	1 μl
DEPC H_2O (0.01%)	to 10 μl
Total volume	10 μl

Table 18. Reaction master mixture

Reagents	Amount
10× RT buffer	2 μl
25 mM $MgCl_2$	4 μl
0.1 M DTT	2 μl
RNase	1 μl
DEPC H_2O (0.01%)	1 μl
Total volume	10 μl

(5) Procedure of RT-PCR using SYBR Green

A. Normalize primer concentrations and mix gene-specific forward and reverse primer pair. Each primer (forward or reverse) concentration in the mixture is 5 pmol/μl.
B. Set up the experiment and the following PCR program on ABI Prism SDS 7000. Do not click on the dissociation protocol if it needs to check the PCR result by agarose gel. Save a copy of the setup file and delete all PCR cycles (used for later dissociation curve analysis).
    a. 50°C 2 min, 1 cycle
    b. 95°C 10 min, 1 cycle
    c. 95°C 15 s -> 60°C 30 s -> 72°C 30 s, 40 cycles
    d. 72°C 10 min, 1 cycle
C. An RT-PCR reaction mixture can be either 50 μl or 25 μl (Table 19).
D. After PCR is finished, remove the tubes from the machine. The PCR specificity is examined by 3% agarose gel using 5 μl from each reaction.
E. Put the tubes back in SDS 7000 and perform dissociation curve analysis with the saved copy of the setup file.
F. Analyze the RT-PCR result with the SDS 7000 software. Check to see if there is any bimodal dissociation curve or abnormal amplification plot.

Table 19. RT-PCR reaction mixture using SYBR Green Mix

Reagents	Reagent Amount (μl)	Reagent Amount (μl)
SYBR Green Mix (2×)	25	12.5
Tissue cDNA	0.5	0.25
Primer pair mix (5 pmol/μl each primer)	2	1
DEPC H_2O (0.01%)	22.5	11.25
Total	50	25

One-step RT-PCR plays reverse transcription and PCR in a single buffer system and in one tube. In two-step RT-PCR, these two steps are performed separately in different tubes.

(6) General recommendations for RT-PCR

A. Use positive-displacement pipettes to avoid inaccuracies in pipetting.
B. The sensitivity of RT-PCR allows detection of the target in 2 pg of total RNA. The number of copies of total RNA used in the reaction should ideally be enough to give a signal by 25-30 cycles (preferably less than 100 ng). The amount used should be decreased or increased to achieve this.
C. The optimal concentrations of the reagents are as follows:
    a. $MgCl_2$ concentration should be between 4 and 7 mM.
    b. Concentrations of dNTPs should be balanced with the exception of dUTP (if used). Substitution of dUTP for dTTP for control of PCR product carryover requires twice dUTP that of other dNTPs. While the optimal range for dNTPs is 0.5-1 mM (for one-step RT-PCR), for a typical TaqMan reaction 0.2 mM of each dNTP (0.4 mM of dUTP) is suitable.
    c. 250 nl (1.25 units) AmpliTaq DNA polymerase (5.0 units/μl) is added into each 0.05 ml reaction. This is the minimum requirement. Optimisation can be done by increasing this amount by 0.25 unit increments.
    d. Optimal probe concentration is 50-200 nM, and the primer concentration is 100-900 nM. Ideally, each primer pair should be optimised at three different temperatures (58, 60 and 62^0C for TaqMan primers) and at each combination of three concentrations (50, 300, 900 nM). This means setting up three different sets (for three temperatures) with nine reactions in each (50/50 mM, 50/300 mM, 50/900, 300/50, 300/300, 300/900, 900/50, 900/300, 900/900 mM) using a fixed amount of target template. Optimal performance is achieved by selecting the primer concentrations that provide the lowest C_T and highest ΔRn. Similarly, probe concentration should be optimised for 25-250 nM.
D. If AmpliTaq Gold DNA polymerase is used, there has to be a 9-12 min pre-PCR heat step at 92-95°C to activate it. If AmpliTaq Gold DNA polymerase is used, there is no need to set up the reaction on ice. A typical TaqMan reaction consists of 2 min at 50°C for uracil-N-glycosylase (UNG) incubation, 10 min at 95°C for Polymerase activation, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. A typical reverse transcription cycle, which should precede the TaqMan reaction if the starting

material is total RNA, consists of 10 min at 25°C, 30 min at 48°C and 5 min at 95°C.

E. AmpErase UNG is added in the reaction to prevent the reamplification of carry-over PCR products by removing any uracil incorporated into amplicons. This is why dUTP is used rather than dTTP in PCR reaction. UNG does not function above 55°C and does not cut single-stranded DNA with terminal dU nucleotides. UNG-containing master mix should not be used with one-step RT-PCR unless rTth DNA polymerase is being used for reverse transcription and PCR.

F. It is necessary to include at least three No Amplification Controls (NAC, a minus-reverse transcriptase control) as well as three No Template Controls (NTC, a minus sample control) in each reaction plate to achieve a 99.7% confidence level in the definition of +/- thresholds for the target amplification, six replicates of NTCs must be run. NAC is a mock reverse transcription containing all the RT-PCR reagents, except the reverse transcriptase; NTC includes all of the RT-PCR reagents except the RNA template.

G. The dynamic range of a primer/probe system and its normaliser should be examined if the $\Delta\Delta C_T$ method is going to be used for relative quantitation. The linear dynamic range refers to the range of initial template concentrations over which accurate C_T values are obtained. This is determined by running reactions of five RNA concentrations (for example, 0, 80 pg/μl, 400 pg/μl, 2 ng/μl and 50 ng/μl). The resulting plot of log of the initial amount vs C_T values (standard curve) should be a (near) straight line for both the target and normaliser RT-PCRs for the same range of total RNA concentrations.

H. The passive reference is a dye (ROX) included in the reaction (present in the TaqMan universal PCR master mix). It does not participate in the 5' nuclease reaction. It provides an internal reference for background fluorescence emission. This is used to normalise the reporter-dye signal. This normalisation is for non-PCR-related fluorescence fluctuations occurring in different wells or over time and different from the normalisation for the amount of cDNA or efficiency of the PCR. Normalisation is achieved by dividing the emission intensity of reporter dye by the emission intensity of the passive reference. This gives the ratio defined as Rn.

I. In addition to the use of ROX, a master mix should be used when setting up multiple reactions to minimize sample-to-sample and well-to-well variation and improve reproducibility (ROX will be within the master mix).

J. If multiplexing is done, the more abundant of the targets will use up all the ingredients of the reaction before the other target gets a chance to amplify. To avoid this, the primer concentrations for the more abundant target should be limited.

K. If SYBR green is used, dissociation (melting) curve analysis should be performed. Ideally, the experimental samples should yield a sharp peak (first derivative plot) at the T_m of the amplicon, whereas the NAC and NTC will not generate significant fluorescent signal. This result indicates that the products are specific, and that SYBR Green I fluorescence is a direct measure of accumulation of the product of interest. If the dissociation curve has a series of peaks, there is not enough discrimination between specific and non-specific reaction products. To obtain meaningful data, optimisation of the RT-PCR would be necessary.

(7) Recommendations for the general assay of cDNA samples

A. Reverse transcription of total RNA to cDNA should be done with random hexamers. If oligo-dT has to be used long mRNA transcripts or amplicons greater than 2 kbp upstream should be avoided, and 18S RNA cannot be used.

B. Multiplex PCR will only work properly if the control primers are limiting (ABI control reagents do not have their primers limited). This requires running primer limiting assays for optimisation.

C. Range of target cDNA used is 10-1000 ng.

D. It is ideal to treat each RNA preparation with RNase free DNase to avoid genomic DNA contamination.

E. The reagents and the PCR mixture itself should be vortexed and mixed well. Otherwise there may be shifting Rn values during the early cycles (0-5) of PCR. Add probe to buffer and allow it to equilibrate at room temperature prior to reagent mix formulation.

(8) Setting up one-step TaqMan reaction

One-step RT-PCR uses RNA as a template. This is the preferred method if the RNA solution has a low concentration. The disadvantage is that RNA carryover prevention enzyme AmpErase cannot be used in one-step reaction format. In this method, both reverse transcriptase and RT-PCR take place in the same tube. The downstream PCR primer also acts as the primer for reverse transcriptase (random hexamers or oligo-dT cannot be used for reverse transcription in one-step RT-PCR). One-step reaction requires higher dNTP concentration (≥300 mM) as it combines two reactions needing dNTPs in one. A typical reaction mix for one-step PCR by Gold RT-PCR kit is as follows (Table 20):

Table 20. A typical reaction mix for one-step PCR by Gold RT-PCR kit

Reagents	Amount (μl)	Final Concentration (μM)
H_2O + RNA	20.5	RNA 10 pg-100 ng
10× TaqMan buffer	5	1×
$MgCl_2$ (25 mM)	11	5500
dATP (10 mM):	1.5	300
dCTP (10 mM)	1.5	300
dGTP (10 mM)	1.5	300
dUTP (20 mM)	1.5	600
Primer F (10 μM)	2.5	0.5
Primer R (10 μM)	2.5	0.5
TaqMan Probe	1	0.1
AmpliTaq Gold	0.25	
Reverse Transcriptase	0.25	
RNase inhibitor	1	
Total	50	

10 pg-100 ng RNA will be used in this reaction. Decreasing the amount of template from 100 ng to 50 ng will increase the C_T value by 1. To decrease a C_T value by 3, the initial amount of template should be increased 8-fold. ABI claims that 2 pg RNA can be detected by this system and the maximum amount of RNA that can be used is 1 mg. For routine analysis, 10 pg-100 ng RNA and 100 pg-1000 ng genomic DNA can be used.

(9) Cycling parameters for one-step PCR

- Reverse transcription (by MuLV) 48^0C for 30 min
- AmpliTaq activation 95^0C for 10 min
- PCR will run denaturation at 95^0C for 15 sec and annealing/extension at 60^0C for 1 min (repeated 40 times). There are only two steps in a RT-PCR cycle.
- On ABI 7700, minimum holding time is 15 seconds.

(10) Operating ABI 7700 produced by Applied Biosystems, Inc.

Make sure the following before starting a run:

A. Cycle parameters are correct for the run.
B. Choice of spectral compensation is correct.
C. Choice of "Number of PCR Stages" is correct in the Analysis Options box.
D. No Template Control (NTC) is labelled.
E. The choice of dye component should be made correctly before data analysis.
F. Save the run before it starts by giving it a name.
G. The ABI software requires extreme caution. Do not attempt to stop a run after clicking on the Run button.

When analysing the data, the default setting for baseline fluorescence calculation is 3-15 cycles. If any C_T value is <15, the baseline should be changed accordingly to obtain accurate C_T values the baseline stop cycle needs to be set two cycles earlier than the C_T value for the most abundant sample. If the results do not make sense, check the raw spectra for a possible CDC camera saturation during the run. Saturation of CDC camera may be prevented by using optical caps rather than optical adhesive cover. It is also more likely to happen when SYBR Green I is used, when multiplexing and when a high concentration of probe is used.

(11) Interpretation of results

At the end of each reaction, the recorded fluorescence intensity is used for the following calculations by the software of the system used: Rn^+ is the Rn value of a reaction containing all

components. Rn^- is the Rn value detected in NTC. ΔRn is the difference between Rn^+ and Rn^-. It is an indicator of the magnitude of the signal generated by the PCR. It is the ΔRn plotted against cycle numbers that produces the amplification curves and gives the C_T value.

There are different approaches to quantitate the amount of template (Livak, 2001):

A. **Absolute standard curve method:** Absolute quantification determines the input copy number of the transcript of interest, usually by relating the PCR signal to a standard curve. In this method, a standard curve is first constructed from RNA of known concentration. This curve is then used as a reference standard for extrapolating quantitative information for mRNA targets of unknown concentrations. cDNA plasmids are the preferred standards for absolute quantitation. This method has been used to estimate cytokine concentrations (Giulietti, 2001), CMV (Kearn, 2001a; Kearns, 2001b; Kearns, 2002; Mengelle, 2003), HIV (Gibellini, 2004) and other viral loads (Niesters, 2001), (Saha, 2001). See Bustin, 2000 for a review and Absolute Quantification Page by Pfaffl.

B. **Relative standard method (relative fold change):** In this method, one of the experimental samples is the calibrator, or 1× sample. Each of the normalised target values is divided by the calibrator normalised target value to generate the relative expression levels. Target quantity is determined from the standard curve and divided by the target quantity of the calibrator. The calibrator is the 1× sample, and all other quantities are expressed as an n-fold difference relative to the calibrator. The calibrator is usually the expression level at baseline and the experimental samples are those collected after treatment or some intervention. The calibrator should be available at large enough quantities to be included in each run.

(12) Comparative threshold method

This method uses no known amount of standard but compares the relative amount of the target sequence to any of the reference values chosen and the result is given as relative to the reference value (such as the expression level of resting lymphocytes or a standard cell line or in comparison to the baseline value). For the C_T calculation to be valid, the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal. A sensitive method for assessing if two amplicons have the same efficiency is to look at how C_T varies with template dilution. Before using the $\Delta\Delta C_T$ method for quantitation, a validation experiment is performed to demonstrate that efficiencies of target and reference are approximately equal. Serial dilutions of the target and normaliser are prepared and RT-PCR is run in separate tubes. The C_T values for each dilution of the target and the normaliser are obtained and their difference for each dilution is calculated (ΔC_T). Then, a plot of log input (like from 0.01 ng to 1 ng) amount versus ΔC_T is prepared. If the efficiencies of the two amplicons are approximately equal, the plot of log input amount versus ΔC_T has a slope of approximately zero (the absolute value of the slope of log input amount vs C_T should be < 0.1). This method has been used in monitoring the immune system activity after transplantation (Sabek, 2002).

A. The comparative C_T method ($\Delta\Delta C_T$) for relative quantitation of gene expression

This method enables relative quantitation of template and increases sample throughput by eliminating the need for standard curves when looking at expression levels relative to an active reference control (normaliser). For this method to be successful, the dynamic range of both the target and reference should be similar. A sensitive method to control this is to look at how ΔC_T (the difference between the two CT values of two PCRs for the same initial template amount) varies with template dilution. If the efficiencies of the two amplicons are approximately equal, the plot of log input amount versus ΔC_T will have a nearly horizontal line (a slope of <0.10). This means that both PCRs perform equally efficiently across the range of initial template amounts. If the plot shows unequal efficiency, the standard curve method should be used for quantitation of gene expression. The dynamic range should be determined for both (1) minimum and maximum concentrations of the targets for which the results are accurate and (2) minimum and maximum ratios of two gene quantities for which the results are accurate. In conventional competitive RT-PCR, the dynamic range is limited to a target-to-competitor ratio of about 10:1 to 1:10 (the best accuracy is obtained for 1:1 ratio). The RT-PCR is able to achieve a much wider dynamic range.

Running the target and endogenous control amplifications in separate tubes and using the

standard curve method requires the least amount of optimisation and validation. The advantage of using the comparative C_T method is that the need for a standard curve is eliminated (more wells are available for samples). It also eliminates the adverse effect of any dilution errors made in creating the standard curve samples.

As long as the target and normaliser have similar dynamic ranges, the comparative C_T method ($\Delta\Delta C_T$ method) is the most practical method. It is expected that the normaliser will have a higher expression level than the target (thus, a smaller C_T value). The calculations for the quantitation start with getting the difference (ΔC_T) between the C_T values of the target and the normaliser:

$\Delta C_T = C_T$ (target) - C_T (normaliser/calibrator/reference)

This value is calculated for each sample to be quantitated. One of these samples should be chosen as the reference for each comparison to be made. The comparative $\Delta\Delta C_T$ calculation involves finding the difference between each sample's ΔC_T and the baseline's ΔC_T. If the baseline value is representing the minimum level of expression, the $\Delta\Delta C_T$ values are expected to be negative (because the ΔC_T for the baseline sample will be the largest as it will have the greatest C_T value). If the expression is increased in some samples and decreased in others, the DDC_T values will be a mixture of negative and positive ones. The last step in quantitation is to transform these values to absolute values. The formula for this is: comparative expression level=$2^{-\Delta\Delta Ct}$.

For expressions increased compared to the baseline level this will be something like 2^3=8 times increase, and for decreased expression it will be something like 2^{-3} = 1/8 of the reference level. Microsoft Excel can be used to do these calculations by simply entering the C_T values. A more accurate method of relative quantification using the relative expression ratio is presented by Pfaffl (Pfaffl, 2001).

B. Points to remember and trouble shooting

a. TaqMan Universal PCR master mix should be stored at 2-8^{0}C (not at -20^{0}C).
b. The GAPDH probe supplied with the TaqMan Gold RT-PCR kit is labelled with a JOE reporter dye, the same probe provided within the Pre-Developed TaqMan Assay Reagents kit is labelled with VIC. Primers for these human GAPDH assays are designed not to amplify genomic DNA.
c. Carryover prevention enzyme, AmpErase UNG, cannot be used with one-step RT-PCR which requires incubation at 48^{0}C but may be used with the EZ RT-PCR kit.
d. It is ideal to run duplicates to control pipetting errors but this inevitably increases the cost.
e. If multiplexing, the spectral compensation option (in Advanced Options) should be checked before the run.
f. Normalisation for the fluorescent fluctuation by using a passive reference (ROX) in the reaction and for the amount of cDNA/PCR efficiency by using an endogenous control (such as GAPDH, active reference) is different processes.
g. ABI 7700 can be used not only for quantitative RT-PCR but also end-point PCR. The latter includes presence/absence assays or allelic discrimination assays such as SNP typing.
h. Shifting Rn values during the early cycles (cycle 0-5) of PCR means initial disequilibrium of the reaction components and does not affect the final results as long as the lower value of baseline range is reset.
i. If an abnormal amplification plot has been noted (C_T value <15 cycles with amplification signal detected in early cycles), the upper value of the baseline range should be lowered and the samples should be diluted to increase the C_T value (a high C_T value may also be due to contamination),
j. A small ΔRn value (or greater than expected C_T value) indicates either poor PCR efficiency or low copy number of the target. This may also occur in the case of contamination of NTC.
k. A standard deviation >0.16 for C_T value indicates inaccurate pipetting.
l. SYBR Green entry in the Pure Dye Setup should be abbreviated as "SYBR" in capitals. Any other abbreviation or lower case letters will cause problems.
m. The ABI 7700 should not be deactivated for extended periods of time. If it has ever been shutdown, it should be allowed to warm up for at least one hour before a run. Leaving the instrument on all times is recommended and is beneficial for the laser. If the machine has been switched on just before a run, an error box stating a firmware version conflict may appear. If

this happens, choose the "Auto Download" option.

n. The ABI 7700 is only one of the many RT-PCR systems in a very competitive market.

(13) Advantages of using RT-PCR

A. Traditional PCR is measured at end-point (plateau), while RT-PCR collects data in the exponential growth phase
B. An increase in reporter fluorescent signal is directly proportional to the number of amplicons generated
C. The cleaved probe provides a permanent record amplification of an amplicon
D. Increased dynamic range of detection
E. Requirement of 1000-fold less RNA than conventional assays
F. No-post PCR processing due to closed system (no electrophoretical separation of amplified DNA)
G. Detection is capable down to a 2-fold change
H. Small amplicon size results in increased amplification efficiency

(14) RT-PCR Applications

RT-PCR can be applied to traditional PCR applications as well as new applications that would have been less effective with traditional PCR. With the ability to collect data in the exponential growth phase, the power of PCR has been expanded into applications such as:

A. Quantitation of gene expression (Giulietti, 2001) including NK cell KIR gene expression (Leung, 2005)
B. Array verification (Rajeevan, 2001). See also Verification of Array Results Page by Pfaffl.
C. Biosafety and genetic stability testing (Lovatt, 2002)
D. Drug therapy efficacy / drug monitoring (Leruez-Ville, 2004; Brennan, 2003; Burger, 2003; Kogure, 2004)
E. Real-Time Immuno-PCR (IPCR) (Adler, 2003; Barletta, 2004; Lind & Kubista, 2005)
F. Viral quantitation (Niesters, 2001; Mengelle, 2003)
G. Pathogen detection (Belgrader, 1999; Uhl, 2002; Mackay, 2004; Perandin, 2004; Watzinger, 2004) including CMV detection (Kearns, 2001a; Kearns, 2001b; Kearns, 2002; Mengelle, 2003), rapid diagnosis of meningococcal infection (Bryant, 2004), penicillin susceptibility of Streptococcus pneumoniae (Kearns, 2002), Mycobacterium tuberculosis and its resistant strains (Kraus, 2001; Torres, 2003; Cleary, 2003; Hazbon, 2004), and waterborne microbial pathogens in the environment (Foulds, 2002; Guy, 2003)
H. DNA damage (microsatellite instability) measurement (Dietmaier, 2001)
I. Radiation exposure assessment (Blakely, 2001; Blakely, 2002; Grace, 2002; Grace, 2003)
J. In vivo imaging of cellular processes (Tung, 2000; Bremer, 2002)
K. Mitochondrial DNA studies (He, 2002; Liu, 2003; Alonso, 2004)
L. Methylation detection (Trinh, 2001; Cottrell, 2004; Lehmann, 2004; Thomassin, 2004)
M. Detection of inactivation at X-chromosome (Hartshorn, 2002; van Dijk, 2002)
N. Determination of identity at highly polymorphic HLA loci (Zhou, 2004)
O. Monitoring post transplant solid organ graft outcome (Sabek, 2002; Gibbs, 2003)
P. Monitoring chimerism after haematopoietic stem cell transplantation (Elmaagacli, 2002; Alizadeh, 2002; Thiede, 2004; Harries, 2004)
Q. Monitoring minimal residual disease after haematopoietic stem cell transplantation (Elmaagacli, 2002; Cilloni, 2002; Sarris, 2002; Gabert, 2003; Van der Velden, 2003)
R. Genotyping by fluorescence melting-curve analysis (FMCA) or high-resolution melting analysis (HRMA) (von Ahsen 2000; Donohoe, 2000; Lyon, 2001; Waterfall & Cobb, 2002; Bennett, 2003; Wittwer, 2003; Zhou, 2005; Palais, 2005; Chou, 2005) or specific probes/beacons (Tapp, 2000; Mhlanga, 2001; Solinas, 2001; Song, 2002; Gupta, 2004; reviewed in Lareu, 2004)
    a. Trisomies (Zimmermann, 2002) and single-gene copy numbers (Bieche, 1998; Mocellin, 2003, Barrois, 2004; Linzmeier, 2005).
    b. Microdeletion genotypes (Laurendeau, 1999; Kariyazono, 2001; Covault, 2003; Coupry, 2004)
    c. Haplotyping (Von Ahsen, 2004)
    d. Quantitative microsatellite analysis (Ginzinger, 2000)
    e. DNA pooling and quantitative allelic discrimination (Barcellos, 2001; Abbas, 2004; Quesada, 2004)

f. Prenatal diagnosis/sex determination using single cell isolated from maternal blood (Hahn, 2000; Bischoff, 2002; Bischoff, 2003) or fetal DNA in maternal circulation (Bischoff, 2002; Hwa, 2004)
g. Prenatal diagnosis of haemoglobinopathies (Kanavakis, 1997; Vrettou, 2003; Vrettou, 2004)
h. Intraoperative cancer diagnostics (Raja, 2002)

S. Linear-after-the-exponential (LATE)-PCR: a new method for real-time quantitative analysis of target numbers in small samples, which is adaptable to high throughput applications in clinical diagnostics, biodefense, forensics, and DNA sequencing (Sanchez, 2004).

From the name we can see that RT-PCR is a technique to detect the progress of a PCR reaction in real time. At the same time, a relatively small amount of PCR product (DNA, cDNA or RNA) can be quantified. RT-PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green) or sequence specific probes (i.e. Molecular Beacons or TaqMan® Probes). RT-PCR facilitates the monitoring of the reaction as it progresses. It can start with minimal amounts of nucleic acid and quantify the end product accurately. Moreover, there is no need for the post PCR processing which saves the resources and the time. These advantages of the fluorescence based QPCR technique have completely revolutionized the approach to PCR-based quantification of DNA and RNA. Real-time assays are now easy to perform, have high sensitivity, more specificity, and provide scope for automation.

There are numerous potential applications for RT-PCR. Normally we want to know how the genetic expression of a particular gene changes over time, such as during germination, or in response to changes in environmental conditions. RT-PCR has been used to detect changes in gene expression in a tissue in response to an administered pharmacological agent and is thus an important technique in drug discovery and testing. In recent years, RT-PCR has been slightly superseded by DNA microarray technology, which allows the expression of many genes to be quantified in a cell sample instead of just one. However, a standard RT-PCR experiment is still cheaper and easier to set up than an average microarray, and so remains an important tool in molecular biology labs (Ma, 2005).

One reason that makes reverse transcriptase-PCR non-quantitative is that ethidium bromide is a rather insensitive stain. Methods such as competitive PCR are developed to make the method more quantitative but they are very cumbersome and time-consuming to perform. Thus, RT-PCR (or reverse transcriptase RT-PCR) was developed.

RT-PCR has simplified and accelerated PCR laboratory procedures and has increased information obtained from specimens including routine quantification and differentiation of amplification products. Clinical diagnostic applications and uses of RT-PCR are growing exponentially, RT-PCR is rapidly replacing traditional PCR, and new diagnostic uses likely will emerge (Kaltenboeck, 2005).

RT-PCR can be used for quantitative or qualitative evaluation of PCR products and is ideally suited for analysis of nucleotide sequence variations (point mutations) and gene dosage changes (locus deletions or insertions/duplications) that cause human monogenic diseases. RT-PCR offers a means for more rapid and potentially higher throughput assays, without compromising accuracy and has several advantages over end-point PCR analysis, including the elimination of post-PCR processing steps and a wide dynamic range of detection with a high degree of sensitivity. This review will focus on RT-PCR protocols that are suitable for genotyping monogenic diseases with particular emphasis on applications to prenatal diagnosis, non-invasive prenatal diagnosis and preimplantation genetic diagnosis (Traeger-Synodinos, 2006). According to Wortmann et al study, RT-PCR offers rapid (within hours) identification of Leishmania to the complex level and provides a useful molecular tool to assist both epidemiologists and clinicians **(**Wortmann, 2005**).**

Reverse transcription PCR is also a basic technique for molecular biological research and it ahs a widely usage in the life sciences. The exponential amplification of complementary sequence of mRNA or RNA sequences via reverse transcription PCR allow for a high sensitivity detection technique, where low copy number or less abundant RNA molecules can be detected. It is also used to clone mRNA sequences in the form of complementary DNA, allowing libraries of cDNA (cDNA libraries) to be created which contain all the mRNA sequences of genes expressed in a cell. Furthermore, it allows the creation of cDNA constructs which were cloned by reverse

transcription PCR and allow the expression of genes at the RNA and protein levels for further study.

Combined with Western blot, ELISA and microarray methods, RT-PCT will be very benefit on the gene expression studies (Ma, 2006).

Summarily, the applications of RT-PCR include **(**PREMIER Biosoft International, 2006**):**

A. Quantitative gene expression studies (mRNA synthesis) (qPCR).
B. DNA copy number measurements in genomic or viral DNAs.
C. Allelic discrimination assays or SNP genotyping.
D. Microarray result verifications. .
E. Drug designs.
F. Drug therapy efficacy exploring.
G. DNA damage measurements.

(15) RT-PCR troubleshooting

A. **Little or no PCR product**. Poor quality of PCR templates, primers, or reagents may lead to PCR failures. First, please include appropriate PCR controls to eliminate these possibilities. Some genes are expressed transiently or only in certain tissues. In our experience, this is the most likely cause for negative PCR results. Please read literature for the gene expression patterns. One caveat is that microarrays are not always reliable at measuring gene expressions. After switching to the appropriate templates, we obtained positive PCR results in contrast to the otherwise negative PCRs.
B. **Poor PCR amplification efficiency.** The accuracy of RT-PCR is highly dependent on PCR efficiency. A reasonable efficiency should be at least 80%. Poor primer quality is the leading cause for poor PCR efficiency. In this case, the PCR amplification curve usually reaches plateau early and the final fluorescence intensity is significantly lower than that of most other PCRs. This problem may be solved with re-synthesized primers.
C. **Primer dimer.** Primer dimer may be occasionally observed if the gene expression level is very low. If this is the case, increasing the template amount may help eliminate the primer dimer formation. Carefully designed primers will help to limited this problem that requires: limited length of primer in 18 to 24 bps; 50 to 60 % overall GC content; limit structures of G or G's longer than 3 bases; no Gs on the 5' end; limited self binding structure and self pine formation.
D. **Multiple bands on gel or multiple peaks in the melting curve.** Agarose gel electrophoresis or melting curve analysis may not always reliably measure PCR specificity. From our experience, bimodal melting curves are sometimes observed for long amplicons (>200 bp) even when the PCRs are specific. The observed heterogeneity in T_m is due to internal sequence inhomogeneity (e.g. independently melting blocks of high and low GC content) rather than non-specific amplicon. On the other hand, for short amplicons (< 150 bp) very weak (and fussy) bands migrating ahead of the major specific bands are sometimes observed on agarose gel. These weak bands are super-structured or single-stranded version of the specific amplicons in equilibrium state and therefore should be considered specific. Although gel electrophoresis or melting curve analysis alone may not be 100% reliable, the combination of both can always reveal PCR specificity in our experience.
E. **Non-specific amplicons**. Non-specific amplicons, identified by both gel electrophoresis and melting curve analysis, give misleading RT-PCR result. To avoid this problem, please make sure to perform hot-start PCR and use at least 60°C annealing temperature. We noticed not all hot-start Taq polymerases are equally efficient at suppressing polymerase activity during sample setup. The SYBR Green PCR master mix described here always gives us satisfactory results. If the non-specific amplicon is persistent, you have to choose a different primer pair for the gene of interest (PrimerBank, 2006).

8. Kary Banks Mullis Introduce

Kary Banks Mullis (born on December 28, 1944) is an American biochemist who developed the PCR, the central technique in molecular biology which allows the amplification of specified DNA sequences, for which he was awarded the Nobel Prize in Chemistry and the Japan Prize in 1993. PCR remains one of the most influential molecular techniques of our day, opening the door to a wide variety of genetic and molecular research as well as applications in medicine, forensics, paleontology and other fields. Mullis continues his scientific

work, but has more recently become known for idiosyncratic and sometimes controversial views on topics unrelated to biochemistry.

1) Personal history

Mullis was born in Lenoir, North Carolina, and grew up in Columbia, South Carolina where he attended Dreher High School. He attended the Georgia Institute of Technology, and received a Ph.D. in biochemistry from the University of California, Berkeley in 1973. He has been married four times (including his current marriage), and has two sons and one daughter. He currently resides in Newport Beach, California and in Anderson Valley, California.

2) Inventions

In 1983, while working for the biotechnology company Cetus, Mullis came up with the idea of using the *Thermophilus Aquaticus* (Taq) DNA polymerase to amplify segments of DNA. DNA amplification *in vitro* existed prior to PCR, however the polymerases used were destroyed when the DNA was melted and needed to be repeatedly replaced. The Taq polymerase was heat resistant and only needed to be added once thus making the technique dramatically more cost effective. A more complete story of the development of this technology can be found elsewhere in Wikipedia: in an article on the source of the enzyme and an article on the history of the technique. The importance of this invention and variations on it can hardly be overstated. It has created revolutions in molecular biology, genetics, medicine and forensics.

Some controversy surrounds the balance of credit that should be given to Mullis versus the team at Cetus; in practice, credit has accrued to both the inventor and the company in the form of a Nobel Prize and a $10000 Cetus bonus for Mullis, and $300 million for Cetus when the company sold the patent to Roche Molecular Systems.

Mullis has also invented a UV-sensitive plastic that changes color in response to light, and most recently has been working on an approach for mobilizing the immune system to neutralize invading pathogens and toxins, leading to the formation of his current venture, Altermune LLC. In his Nobel page, he described this idea this way:

It is a method using specific synthetic chemical linkers to divert an immune response from its nominal target to something completely different which you would right now like to be temporarily immune to. Let's say you just got exposed to a new strain of the flu. You're already immune to alpha-1,3-galactosyl-galactose bonds. All humans are. Why not divert a fraction of those antibodies to the influenza strain you just picked up. A chemical linker synthesized with an alpha-1,3-gal-gal bond on one end and a DNA aptamer devised to bind specifically to the strain of influenza you have on the other end, will link anti-alpha-Gal antibodies to the influenza virus and presto, you have fooled your immune system into attacking the new virus.

3) Public controversies

Some of the principles of PCR were described in 1971 by Kjell Kleppe, a Norwegian scientist, and some have asserted that Kleppe has a better claim to the invention. In addition, the suggestion that Mullis was solely responsible for the idea of using Taq polymerase in the PCR process has been refuted by his co-workers at the time. Indeed, one story is that David Gelfand and Randy Saiki at Cetus developed the idea of using a thermostable DNA polymerase, and that Mullis was only recognized as an inventor after he came to the Cetus Labs wielding a gun. The Kleppe story may best illustrate the fact that an invention, even if has been conceptualized earlier, does not really exist until it can be developed and realized.

The anthropologist Paul Rabinow wrote a book on the history of the PCR method in 1996 which questioned whether or not Mullis "invented" PCR or "merely" came up with the concept of it. Rabinow, a Foucault scholar interested in issues of the production of knowledge, used the topic to argue against the idea that scientific discovery is the product of individual work, writing, "*Committees and science journalists like the idea of associating a unique idea with a unique person, the lone genius. PCR is, in fact, one of the classic examples of teamwork."*

Mullis has also drawn controversy for his past association with Peter Duesberg and his skepticism about the evidence for the idea that HIV causes AIDS. (For more on this topic, see also AIDS reappraisal and the interviews listed below.) As the recipient of a Nobel Prize for the PCR technique that is used to measure viral load in people with AIDS, he has often been cited by people within the AIDS dissident movement as someone who supports their views.

He also denigrates concern about global warming, denying that it is known to be human caused, and disagrees with the idea that CFCs cause ozone depletion.

Mullis became known to a wider public as a potential forensic DNA analyst and witness for the defense in the OJ Simpson trial. News coverage of Mullis, his activities, and his background was extensive, and the defense moved to prevent cross-examination about his personal life including

"social relationships, domestic discord and use of controlled substances". However, evidently satisfied that they had adequately discredited the DNA evidence without him, the defense did not call him.

In Mullis's 1998 essay collection, *Dancing Naked in the Mind Field*, he relates a number of experiences that some consider strange, and which critics point out to question his scientific judgment. He also details his use of LSD. Perhaps the strangest episode Mullis relates, he says, happened while at his cabin in the remote northern California wilds; John Edward Mack noted that episode has many hallmarks of the abduction phenomenon. Late one evening while walking to the latrine, Mullis saw a "glowing raccoon" which spoke to him, saying "Good evening, doctor." The next thing he remembered, several hours had seemingly passed without his recall: it was dawn, and he was strolling on a path near the cabin. His clothing was dry and clean, unlike what he'd expected from wandering the forest in the dark of night for several hours.

Mullis later wrote, "I wouldn't try to publish a scientific paper about these things, because I can't do any experiments. I can't make glowing raccoons appear. I can't buy them from a scientific supply house to study. I can't cause myself to be lost again for several hours. But I don't deny what happened. It's what science calls anecdotal, because it only happened in a way that you can't reproduce. But it happened." (Wikipedia, 2006).

In the following it gives the PCR inventor Kary Banks Mullis' biography:

- 1944, December 28: Kary Banks Mullis (male) was born on in Lenoir North, North Carolina, USA.
- 1966: received a bachelor degree in chemistry from the Georgia Institute of Technology, USA.
- 1972: obtained his Ph.D. in biochemistry from the Berkeley of University of California, USA.
- 1972-1973: Taught biochemistry at Berkeley Berkeley of University of California, USA.
- 1973-1977: postdoctoral fellow in pediatric cardiology (in the areas of angiotensin and pulmonary vascular physiology) at the University of Kansas Medical School, USA.
- 1977-1979: postdoctoral fellow in pharmaceutical chemistry at the University of California, San Francisco, USA. **1979-1985, Cetus Corp. in Emeryville, California, USA, on the DNA chemistry study. During this period he conducted research on oligonucleotide synthesis and invented PCR.**
- 1986: director of molecular biology at Xytronyx, Inc. in San Diego of California of USA, on DNA technology and photochemistry.
- 1987: began consulting on nucleic acid chemistry for more than a dozen corporations, including Angenics, Cytometrics, Eastman Kodak, Abbott Labs, Milligen/Biosearch and Specialty Laboratories.
- 1990: awarded the William Allan Memorial Award of the American Society of Human Genetics, USA.
- 1990: awarded the Award of Preis Biochemische Analytik of the German Society of Clinical Chemistry and Boehringer Mannheim, German.
- 1991: awarded the National Biotechnology Award, USA.
- 1991: awarded the Gairdner Award, Toronto, Canada.
- 1991: awarded the R&D Scientist of the Year, USA.
- 1992: awarded California Scientist of the Year Award, USA.
- **1993: obtained a Nobel Prize in chemistry for his invention of PCR.**
- 1993: obtained the Japan Prize for the PCR invention, Japan. It is one of international science's most prestigious awards.
- 1993: obtained Thomas A. Edison Award, USA.
- 1994: obtained the honorary degree of Doctor of Science from the University of South Carolina, USA.
- 1998: inducted into the National Inventors Hall of Fame, USA.
- Mullis has several major patents, such as the PCR technology and UV-sensitive plastic that changes color in response to light. His most recent patent application covers a revolutionary approach for instantly mobilizing the immune system to neutralize invading pathogens and toxins, leading to the formation of his latest venture, Altermune LLC.
- His many publications include "The Cosmological Significance of Time Reversal" (Nature), "The Unusual

Origin of the Polymerase Chain Reaction" (Scientific American), "Primer-directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase" (Science), and "Specific Synthesis of DNA In Vitro via a Polymerase Catalyzed Chain Reaction" (Methods in Enzymology). His autobiography, "Dancing Naked in the Mind Field," was published by Pantheon Books in 1998.

- He is currently a Distinguished Researcher at Children's Hospital and Research Institute in Oakland, California. He serves on the board of scientific advisors of several companies, provides expert advice in legal matters involving DNA, and is a frequent lecturer at college campuses, corporations and academic meetings around the world. He is living with his wife, Nancy Cosgrove Mullis, in Newport Beach, California, USA and in Anderson Valley, California, USA.
- Besides the invention of PCR and his biochemistry studies, Dr. Mullis also did a lot of thought in the subjects that cross the scientific principles to parapsychology and global social problems, which we can read from his book "Dancing Naked in the Mind Field" (Figure 6) (Mullis, 1998).
- Dr. Kary Banks Mullis' website is: http://www.karymullis.com.

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