Introduction of PCR and RT-PCR

Ma Hongbao *, Margaret Young **, Zhu Yucui ***, Yang Yan *, Zhu Huaijie ****

* Brookdale University Hospital and Medical Center, Brooklyn, New York 11212, USA, ma8080@gmail.com; ** Cambridge, MA 02138, USA; *** Department of Dermatology, Columbia University Medical Center, 630 West, 168th Street, New York, New York 10032, USA; **** The 2nd Affiliated Hospital of Zhengzhou University, 2 Jingba Road, Zhengzhou, Henan 450014, China. jacksun689@gmail.com, yz81@columbia.edu; 011-86-150-3711-5732

Abstract: The real-time polymerase chain reaction (RT-PCR) is also called quantitative real-time polymerase chain reaction (QRT-PCR) or kinetic polymerase chain reaction (kPCR), which is a technique to simultaneously quantify the DNA molecules. It determines whether a specific DNA sequence is present in the sample and the DNA copy numbers in the sample. It is the real-time version of quantitative polymerase chain reaction (qPCR), as a modification of polymerase chain reaction (PCR). The procedure of RT-PCR follows the regular PCR, but the DNA is quantified after each round of amplification. The common method of quantification is 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 can combine with reverse transcription polymerase chain reaction to quantify messenger RNA (mRNA) at a particular time for in a particular cell or tissue.
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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 is the invention of polymerase chain reaction (PCR) by Kary Banks Mullis (Nobel Prize for Chemistry, 1993), which revolutionized the modern life science technology.

PCR, abbreviation of polymerase chain reaction, is an in vitro technique to synthesize large quantities of a given DNA molecule that separates the DNA into two complementary strands, synthesizes new DNA molecules with uses DNA polymerase and repeats this process fastly. 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 (Figure 1) (Greer, 2006; Mullis, 2006). As a biotechnology company established in Berkeley, California, USA in 1972, Cetus Corporation was the original owner of PCR patent and it was sold to Hoffmann-La Roche Inc. in 1991. Kary Banks Mullis awarded Nobel Prize of chemistry for the invention of PCR in 1993. As a fast gene detection, PCR technique has revolutionized many aspects of life science, such as the diagnosis of genetic defects, the detection of the AIDS virus in human cells, criminologist applications, and fossil studies. Let's thank and remember Dr. Mullis for the invention of PCR when we do PCR.

As a molecular biology technique, PCR replicates DNA enzymatically without using a living organism (*in vitro*). Like DNA amplification in living organisms, PCR allows 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 medical and biological research labs 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. 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.

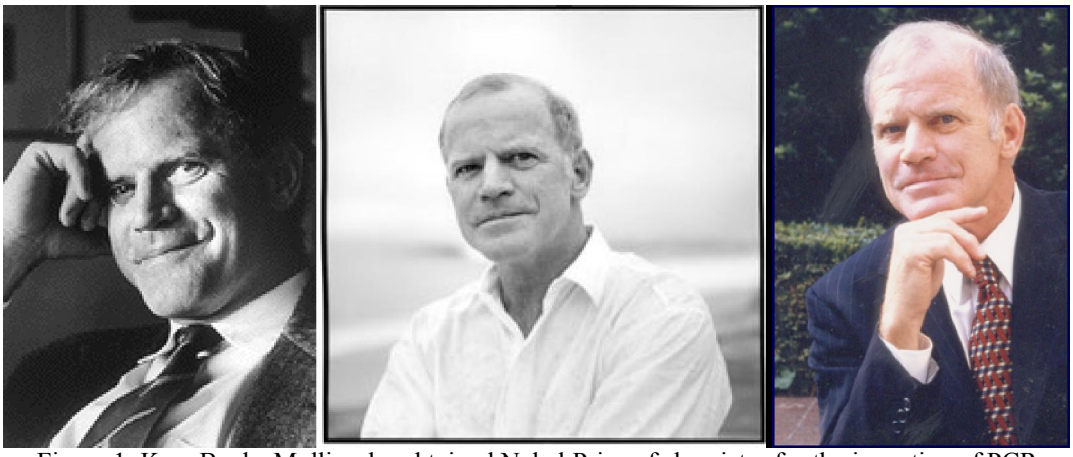

Figure 1. Kary Banks Mullis who obtained Nobel Prize of chemistry for the invention of PCR

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 90°C), annealed to the primers (at 50°C-60°C) and a new strand extended from the primers (72°C). As the new strands themselves act as templates for subsequent cycles, DNA fragments matching both primers are amplified exponentially, rather than linearly. Certain methods can copy fragments up to 47 kb in size, which is still much less than the chromosomal DNA of a eukaryotic cell - for example, a human chromosome contains about three billion base pairs.

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 the DNA amplification is 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 studies 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 *Thermus aquaticus* 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 can be analyzed on an agarose gel and is abundant enough to be detected with ethidium bromide stain. In order to measure messenger RNA (mRNA), the method of 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). Reverse transcriptase PCR analysis of mRNA is often abbreviated as "RT-PCR" also, which is unfortunate as it can be confused with "real-time PCR" that also abbreviated as RT-PCR (Abdul-Careem, 2006**)**. In this article the RT-PCR represents real-time PCR.

Traditionally, PCR 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 sulfur 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 enzyme links the loose nucleotides to the primer and to each of the separated DNA strands in the appropriate sequence. The complete reaction, which takes approximately five minutes, results in two double helices containing the desired portion of the original. The heating and cooling is repeated many times, doubling the number of DNA copies each heating cycle. After thirty to forty 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 bonded ethidium bromide, resulting in an 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 polymerase 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 synthesized. 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 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. Invention of PCR

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 original ownership of PCR patent. Kary Mullis awarded Nobel 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 Pediatric Cardiology and Pharmaceutical Chemistry at the University of Kansas Medical School. After his period at Kansas Medical School he got a technicians 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 license. Universities and companies in other countries are exempt from this patent and are allowed to use the technique without a license.

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.

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. The current opinion is that the inventive property that made the PCR technique was in the idea - the idea created by Kary Banks Mullis.

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 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. And 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 the 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 100 and 0.2 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 (in a controlled environment outside an organism). 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. 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 several hundred to a few thousand 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 patents. There have been several high-profile lawsuits related to the PCR technique, including an unsuccessful lawsuit brought by DuPont (founded in July 1802 as a gun powder plant by Eleuthère Irénée du Pont on Brandywine Creek of Delaware of USA). The pharmaceutical company Hoffmann-La Roche (Founded in 1896 by Fritz Hoffmann-La Roche, Nutley, New Jersey, USA) purchased the rights to the 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). Interestingly, it seems possible that the legal arguments will extend beyond the life of the original PCR and Taq polymerase patents, which expire in 2006.

Since March 28, 2005, it no longer needs a license to practice the basic PCR amplification process, which was covered by U.S. 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 constraints of a license) to people for this specific application. Once Taq DNA polymerase has been incorporated into a scientist's toolbox, it likely remains there for many years, if not for their entire research career. In fact, 33% of respondents have been using Taq polymerase for more than 10 years in their research.

4. Brief History of PCR (Roche Diagnostics, 2006)

1983 Kary Banks Mullis at Cetus Corporation conceived of PCR.

1985 Cetus filed first PCR patent application.

- First publication of PCR by Cetus Corporation appeared in Science. This is the original publication that first described the PCR process, amplification of human beta-globin genes and application to clinical diagnosis.

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

- First forensic use of DNA typing of HLA-DQA locus using PCR in United States.

1987 Cetus was awarded fundamental patents for PCR.

1988 PerkinElmer introduced the automated thermal cycler.

- Science printed first published description of PCR with thermostable polymerase.
- First post-conviction review using PCR on a forensic specimen.

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

- Introduction of AmpliTaq DNA Polymerase (first cloned recombinant *Taq* DNA polymerase).
- 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.

- Dr. D. Gelfand and Ms. S. Stoffel were named Distinguished Inventors for purifying *Taq* DNA polymerase.
- Drs. H. Erlich and K. Mullis received the Biochemical Analysis Award from the German Society of Clinical Chemistry.
- Scientists achieved the first simultaneous amplification and detection of specific DNA sequences 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.

- First publication on r*Tth* and launch of first thermostable RT-PCR research kit.
- First publication describing the technology allowing simultaneous amplification and detection of genetic material later incorporated into TaqMan tests and instruments.
- Dr. H. Erlich received the Advanced Technology in Biotechnology (ATB'91) Milano Award from the International Federation of Clinical Chemistry.
- Hoffmann-La Roche Inc. acquired worldwide rights and patents to PCR.
- Roche Molecular Systems, Inc. (informally called Roche Molecular Diagnostics, or RMD) was founded exclusively to develop diagnostic and other tests utilizing PCR technology.

1992 AMPLICOR Chlamydia trachomatis Test (CT) and AMPLICOR HIV-1 MONITOR Test were introduced outside of the United States.

1993 AMPLICOR HCV MONITOR Test was introduced outside of the United States.
- AMPLICOR CT Test received 510K clearance by the United States Food and Drug Administration (FDA) and launches in the United States, making it the first FDA-cleared PCR test.
- Dr. Kary Banks Mullis obtained Nobel Prize in Chemistry for conceiving PCR technology.

1994 Japanese Red Cross and RMS form partnership to initiate PCR-based testing for screening donated blood for HIV, HCV and HBV.
- *rTth* EZ RT-PCR research kit launches.
- *Tth* XL [Extra Long] DNA PCR research kit launches.
- The first in a series of U.S. 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 the United States.
- AMPLICOR HIV-1 MONITOR Test and AMPLICOR HCV MONITOR Test, the first standardized "quantitative" PCR kits, launch outside of the United States.
- AMPLICOR CT/NG "multiplex" test launches outside of the United States.
- AmpliTaq GOLD DNA polymerase launches, featuring the "Hot Start" form of the enzyme.
- Introduction of AmpliTaq DNA polymerase FS (the enzyme that sequenced the Human Genome).

1996 Introduction of an internal control in AMPLICOR CT/NG Test.
- FDA approves AMPLICOR HIV-1 MONITOR Test.
- FDA clears AMPLICOR MTB Test for detecting Mycobacterium tuberculosis (MTb) DNA.
- RMS scientists describe the first ribonucleotide-incorporating thermostable "designer" DNA Polymerase and "PCR ribo-sequencing."
- Dynal launches DRB-29 HLA-typing kit for tissue typing.

1997 FDA clears COBAS AMPLICOR Analyzer; product is launched in the United States.

1998 FDA clears COBAS AMPLICOR Analyzer for clinical use.
- FDA approves COBAS AMPLICOR Chlamydia trachomatis Detection Test.
- Dynal launches DQB1-25, HLA B-56 and HLA A-35 HLA-typing kits for use in transplantation tissue typing.

1999 FDA approves AMPLICOR HIV-1 MONITOR UltraSensitive Test and clears COBAS AMPLICOR CT/NG Test.
- U.S. blood centers implement 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.
- Dr. Tom White is presented with Caregiver Award from the AIDS Healthcare Foundation.
- Japanese Red Cross Society implements NAT testing to screen 100% of donated blood for HIV, HCV and HBV using AmpliNAT MPX system.
- U.S. patent awarded to RMD inventors for thermostable ribonucleotide incorporating "designer" DNA Polymerase.
- U.S. patent awarded ("the '056 patent") 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 ("real-time PCR" or "kinetic PCR").
- Dynal launches DRB-36 HLA-typing kit for tissue typing.
- LightCycler® TeloTAGGG hTERT Quantification Kit launches.

2000 National Human Genome Research Institute (NHGRI) of the National Institutes of Health (NIH) awards 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 receives 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 launches.
- Dynal launches HLA C-34 typing kit for tissue typing.
- Roche's LinearArray CF Gold, a cystic Fibrosis mutation-detection product, is launched as an Analyte Specific Reagent in the United States.
- Cystic Fibrosis mutation-detection Analyte Specific Reagent launches.
- First publication of the descriptions of "designer" DNA Polymerase with magnesium-activated thermostable reverse transcriptase activity, as well as the first thermostable reverse transcriptase with proofreading activity.

2001 U.S. patent awarded ("the '785 patent") to RMD inventors for a fiber-optic-based PCR device to simultaneously amplify, detect and quantitate nucleic acids ("real-time PCR" or "kinetic PCR").
- COBAS AmpliPrep System launches outside of the United States for research use.
- COBAS AmpliScreen HCV and HIV Test kits launch outside of the United States for use in blood screening.
- Roche and Chiron Corporation reach 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 approves AMPLICOR HCV Test 2.0 and COBAS AMPLICOR HCV Test 2.0.

- Launch of TaqManHCV Analyte Specific Reagent in the United States.

2002

- FDA clears Roche's next generation automated PCR system, the COBAS TaqMan Analyzer, Series 96, for commercial use in the United States.
- COBAS AmpliScreen HIV-1 Test, v1.5 receives registration in Italy.
- Manufacture of 4,000th COBAS AMPLICOR Analyzer.
- FDA approves AMPLICOR HIV-1 MONITOR Test, version 1.5, a test with the ability to measure HIV-1 RNA down to 50 copies/mL and that has the ability to detect and quantify non-B HIV subtypes (Group M subtypes A - G).
- Roche receives FDA clearance for the COBAS AmpliScreen System for use in laboratories testing plasma specimens in the blood screening market. The system automates the sample dilution and pooling procedures as well as the amplification and detection steps for analysis of specimens using the PCR-based nucleic acid amplification methods.
- FDA approves the COBAS AmpliScreen HCV Test, v2.0 and the COBAS AmpliScreen HIV-1 Test, v1.5, the first two blood screening tests designed for use with the COBAS AmpliScreen System. The FDA approves Roche's Blood Licensing Applications (BLAs) for these tests in record time. FDA also clears the COBAS AmpliScreen Data Output Management System (DOMS) for use with the COBAS AmpliScreen System.
- FDA grants an Investigational New Drug Application (IND) for the COBAS AmpliScreen HBV Test in July 2002. In August, Roche begins clinical trials of this test.
- Roche sponsors two-year surveillance program in the U.K. to help ensure future performance of industry's HIV viral load assays.
- Roche Diagnostics and Qiagen partner to develop and commercialize an integrated diagnostic system for hepatitis and HIV PCR testing.
- Roche Diagnostics acquires broad Human Papillomavirus (HPV) patent portfolio from the Institut Pasteur.
- Roche and Innogenetics announce the launch of a first series of new microbiology tests, resulting from the co-operation between the two companies.
- Launch of TaqMan HBV Analyte Specific Reagent in the United States.

2003

- Roche and deCODE genetics announce that deCODE scientists have identified specific variations within a single gene that confer significant increased risk of osteoporosis. Under their alliance to develop and bring to market DNA-based diagnostics, the companies work together to analyzing these and other markers to create a test that can identify individuals who are at a higher risk of developing the disease.
- Roche licenses 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 receives 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 announces that its supplement to the AMPLICOR HIV-1 Test, version 1.5 has been accepted for FDA review. This supplement provides data in support of the automation of the purification step using the COBAS AMPLICOR HIV-1 MONITOR Test, version 1.5. The COBAS AmpliPrep/COBAS AMPLICOR HIV-1 MONITOR Test, version 1.5, was designed to address the increasing needs of clinical laboratories to enhance productivity and reduce operational costs.
- Roche and Epigenomics announce a broad three-year collaboration to develop a range of molecular diagnostic and pharmacogenomic cancer products based on Epigenomics' DNA-methylation technologies.
- Roche Diagnostics receives 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's TaqScreen West Nile Virus Test is found to detect other members of the potentially deadly Japanese Encephalitis virus group in donated blood.
- Roche's TaqScreen West Nile Virus Test used to screen the entire Canadian blood supply.
- 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 Polymerase Chain Reaction (PCR) technology.
- The COBAS TaqMan 48 Analyzer, Roche's clinical real-time PCR instrument, is introduced in the U.S. and Europe. The COBAS TaqMan 48 Analyzer is designed for clinical diagnostics laboratories that are seeking a solution to the complexity of molecular diagnostic testing and require a single, simple system for all of their real-time PCR needs. Real-time PCR delivers increased sensitivity and a wider dynamic range more rapidly than traditional PCR.
- Roche launches the AmpliChip CYP450 microarray, the first product of the Roche/Affymetrix collaboration. The product enables laboratories to detect certain naturally occurring variations in genes that can play a major role in drug metabolism.

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 accepted 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. PCR practice

PCR, as currently practiced, requires several basic components:

1) DNA template, which contains the region of the target DNA fragment to be amplified
2) Two primers, 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
4) Deoxynucleotides-triphosphate (dATP, dTTP, dGTP, dCTP), from which the DNA Polymerase builds the new DNA
5) Buffer, which provides a suitable chemical environment for the DNA polymerase
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. To prevent evaporation of the reaction mixture (typically volumes between 15-100 µl per tube), a heated lid is placed on top of the reaction tubes or a layer of oil is put on the surface of the reaction mixture. These machines cost more than US$2,500 in 2004.

Materials for normal handle 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 10X, usually sold with Taq polymerase or you can make your own)
4) $MgCl_2$ (25 mM is convenient)
5) Taq polymerase
6) dNTPs (2 mM stock) (a 2mM stock of dNTPs means that the final concentration of each dNTP (dATP, dCTP, dGTP, and dTTP) is 2mM -- NOT that all dNTPs together make 2mM. dNTPs come as 100mM stocks -- thaw and add 10µL of each dNTP to 460µL of ddH_20 to make 2mM. Store at -20°C).
7) Sterile distilled water
8) Gloves
9) PCR machine
10) Aerosol tips
11) Ice

The final concentrations of reagents in PCR reactions:

1) Buffer: 1X, usually comes as 10X stock. For 25 µl reactions, this means 2.5 µl.
2) dNTPs: for most general PCR, you want the final concentration to be 200 µM, so a 2 mM stock is essentially 10X -- use 2.5 µl per reaction.
3) Primers: a good place to start with primer concentration is 50 pmol of each primer per reaction. If you don't get your desired product, you can increase to 75 pmol or 100 pmol. This usually does the trick.
4) Template: it's not usually necessary to be incredibly fastidious about how much template you add to a reaction. You can get product with incredibly small amounts of starting DNA. It is OK to make a 3 ml plasmid preparation and use 1/6 of a µl per PCR reaction.
5) $MgCl_2$: this is the greatest variable in PCR. The success of a PCR is very dependent on how much magnesium is present in the reaction. For this reason, it is usually advisable to do a magnesium optimization when performing new PCRs. I could be from 1 mM to 6 mM $MgCl_2$. Since the stock is 25 mM, usually, this means that 1 µl of stock equals 1 mM $MgCl_2$ in a 25 µl reaction.

The cycling reactions

There are three major steps in a PCR, which are repeated for 30 or 40 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, all enzymatic reactions stop (for example: the extension from a previous cycle).
2) **Annealing** at 54°C: The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore.
3) **Extension** at 72°C: This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match, get loose again (because of the higher temperature) and don't give an extension of the fragment.

The bases (complementary to the 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, bases are added complementary to the template).

Before the PCR product is used in further applications, it has to be checked if:

1) There is a product formed: Though biochemistry is an exact science, not every PCR is successful. There is for example a possibility that the quality of the DNA is poor, that 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 bases, but the expected gene should be 1800 bases long. In that case, one of the primers probably fits on a part of the gene closer to the other primer. It is also possible that both primers fit on a totally different gene.
3) Only one band is formed: As in the description above, it is possible that the primers fit on the desired locations, and also on other locations. In that case, you can have different bands in one lane on a gel.

PCR buffers

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

Primers

A DNA synthesis primer is a nucleic acid strand to start the DNA replication. The DNA synthesis needs a primer because most DNA polymerases, enzymes that catalyze the replication of DNA but cannot begin synthesizing a new DNA strand from scratch. 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.

Primer design is significant important for PCR, and a good result depends on good primers. For the primer design, first it needs to get the target DNA sequence (it can be gotten from GenBank) and load the sequence to computer to get primer sequence by the prier design software. The choice of the length of the primers and their melting temperature 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* and break off from the DNA template. The melting temperature (T_M) required increases with the length of the primer. Primers that are too short would anneal at several positions on a long DNA template, which would result in non-specific copies. On the other hand, the length of a primer is limited by the temperature required to melt it. Melting temperatures that are 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 melting temperature between 55°C and 65°C. There are several ways to calculate the primer T_M (A, G, C and T are the number of that nucleotides in the primer, respectively. $[Na^+]$ is the concentration of Na^+ in the PCR vial). The choice of the primer length and melting temperature (T_m) of primers depends on a number of considerations. Up to now, most laboratories do not make primers themselves, but order them by specialized companies.

7. PCR Procedure

Brief Steps of Traditional PCR:

1) The DNA strands are denatured at high temperature, breaking the weak hydrogen bonds that bind one side of the helix to the other and separating the rails of DNA.
2) The temperature is lowered and primers (short bits of DNA) are added. The primers bond to their specific sites.
3) The temperature is brought back up to body temperature and Taq polymerase is added.
4) Repeat step one for n cycles, amplifying the DNA.
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 20-35 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 minutes, but up to 5 minutes. 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 melting temperature (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 minutes.

3) Finally, the DNA polymerase has 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. The time for this step depends both on the DNA polymerase itself and on the length of the DNA fragment to be amplified. As a rule-of-thumb, this step takes 1 minute 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 minutes. This last step is highly recommendable if the PCR product is to be ligated into a T vector using TA-cloning.

Examples

The flowing is given an example for the times and temperatures of PCR program.

The reaction mixture consists of

- 1.0 µl DNA template (100 ng/µl)
- 2.5 µl of primer, 1.25 µl per primer (100 ng/µl)
- 1.0 µl Pfu-Polymerase
- 1.0 µl nucleotides
- 5.0 µl buffer solution
- 89.5 µl water

A 200 µl reaction tube containing the 100 µl mixture is inserted into the thermocycler.

The PCR process consists of the following steps:

1) Initialization. The mixture is heated at 96°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, where it is heated at 96°C for 30 seconds. For each cycle, this is usually enough time for the DNA to denature.
3) Annealing by heating at 68°C for 30 seconds: The primers are jiggling around, caused by the Brownian motion. Short bindings are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the Tm 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 template (because of the higher temperature) and are not extended.

The bases (complementary to the 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, bases are added complementary to the template):

1) Steps 2-4 are repeated 25 times, but with good primers and fresh polymerase, 15 to 20 cycles is sufficient.
2) Mixture is held at 7°C. This is useful if one starts the PCR in the evening just before leaving the lab, so it can run overnight. The DNA will not be damaged at 7°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.

8. RT-PCR

Principle of Methodology of RT-PCR

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. In fact, this technique is sensitive enough to enable quantitation of RNA from a single cell.

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. From the PCR cycle number curves

obtained for each sample, a threshold is defined. 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 measures 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 complementary DNA (cDNA) of each mRNA 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 and include such genes as actin, hypoxanthine-guanine phosphoribosyltransferase (HGP) and glyceraldehyde phospho-dehydrogenase (GAPDH), the endogenous costal to correct for potential variation in RNA loading, cDNA synthesis or efficiency of the amplification reaction. For the RT-PCR principle, more mRNA is in a sample, the earlier it will be detected during repeated cycles of amplification. Many systems produced that amplify DNA with a fluorescent dye. RT-PCR machines can detect the amount of fluorescent DNA and thus the amplification progress. Amplification of a given cDNA over time follows a curve, with an initial flat-phase, 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 used 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 threshold is referred to as the C_T. C_T is threshold cycle, the cycle number at which 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. As an example, suppose that we want to measure the expression level of "Gene-M" in two cell samples. After RT-PCR amplification we finds that in sample 1, Gene-M reaches a pre-determined threshold of detection after 18 cycles, known as the C_T value, where as in sample 2 it does not reach the threshold until 22 cycles. If the housekeeping gene has a C_T value of 17 in both cases then the difference between C_T values, or ΔC_T, will be 1 for sample 1 and 5 for sample 2. In this case Gene-M is more highly expressed in sample 1 than in sample 2.

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

According to chemistries, 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 real-time 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.

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, but a probes costs about US$250).

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.

Scorpions

With Scorpion probes, sequence-specific priming and PCR product detection is achieved 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. This prevents the fluorescence from being quenched and a signal is observed.

SYBR Green

SYBR Green provides the simplest and most economical format for detecting and quantitating PCR products in real-time reactions. SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. 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 non-specific 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, with spurious non-specific background only showing up in very late cycles. SYBR Green is the most economical choice for real-time 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 between specific and non-specific product accumulated during PCR, follow up assays are needed to validate results.

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.

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. These values may be compared between samples for an estimate of the relative expression of target RNA in the samples.

Absolute quantitation, using competitive RT-PCR, measures the absolute amount (copies) of a specific mRNA sequence in a sample. Dilutions of a synthetic RNA (identical in sequence, but slightly

shorter than the endogenous target) 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 the case of 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.

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 — that is, they must not produce additional bands or hybridize to each other. 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 ß-actin, GAPDH mRNAs and 18S rRNA, etc. 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.

For relative RT-PCR data to be meaningful, the 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 the 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 ß-actin and GAPDH. However, because of the abundance of 18S rRNA 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 results from the use of competimers — 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. These kits also include control RNA and an Instruction Manual detailing the series of experiments needed to make relative RT-PCR data significant. For those researchers who have validated ß-actin as an appropriate internal control for their system, the QuantumRNA ß-actin Internal Standards are available.

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. These competitors do not effectively control for variations in the RT reaction or for the amplification efficiency of the specific experimental sequence, as do RNA competitors.

Comparative RT-PCR

While exquisitely sensitive, both relative and competitive methods of qRT-PCR have drawbacks. 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.

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). Isolated RNA could be dissolved in diethyl-pyrocarbonate (DEPC) treated water. RNA isolated 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 copied to cDNA by reverse transcriptase using an oligo dT primer (random oligomers may also be used). 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 more than 90^{o}C (~94^{o}C) so that the two strands separate. 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, especially when RT-PCR is used.
4) The temperature is raised to 72^{o}C and the heat-stable Taq DNA polymerase extends the DNA from the primers. Now we have four cDNA strands (from the original two). These are denatured again at approximately 94^{o}C.
5) Again, the primers are annealed at a suitable temperature (normally between 50^{o}C and 60^{o}C).
6) Taq DNA polymerase binds and extends from the primer to the end of the cDNA strand. There are now eight cDNA strands
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 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. Of the 32 double stranded cDNA molecules at this stage, 75% are the same size, that is the size of the distance between the two primers. The number of cDNA molecules of this size doubles at each round of synthesis (logarithmically) while the strands of larger size only increase arithmetically and are soon a small proportion of the total number of molecules.

After 30 to 40 rounds of synthesis of cDNA, the reaction products are usually analyzed by agarose gel electrophoresis. The gel is stained with EB. This type of agarose gel-based analysis of cDNA products of reverse transcriptase-PCR does not allow accurate quantitation since EB is rather insensitive and when a band is detectable, the logarithmic stage of amplification is over. EB is a dye that binds to double stranded DNA by interpolation (intercalation) 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 EB are used in RT-PCR.

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 EB. 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

However, the perfect standard does not exist; therefore whatever we decide to use as a standard or standards should be validated for your tissue. If possible, we should be able to show that it does not change significantly in expression when your cells or tissues are subjected to the experimental variables you plan to use.

Commonly used standards are:

1) Glyceraldehyde-3-phosphate dehydrogenase mRNA
2) Beta 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)

Standard Curve Method

In this 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. Spectrophotometric measurements at 260 nm 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. However, this can be corrected by normalization to a housekeeping gene.

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 (or calibrator) such as a non-treated sample or RNA from normal tissue. The comparative C_T values of both the calibrator 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 calibrator 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 efficiencies of the target and housekeeping genes are similar. If a housekeeping gene cannot be found whose amplification efficiency is similar to the target, then the standard curve method should be used.

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