

## Application of Real-time Polymerase Chain Reaction (RT-PCR)

Hongbao Ma \*, Kuan-Jiunn Shieh \*\*, Geroge Chen \*, X. Tracy Qiao \*\*\*, Mei-Ying Chuang \*\*

\* Michigan State University, East Lansing, MI 48823, USA  
Telephone: (517) 303-3990; Email: [hongbao@msu.edu](mailto:hongbao@msu.edu)

\*\* Department of Chemistry, Chinese Military Academy, Fengshan, Kaohsiung, Taiwan 830, ROC;  
Telephone: 011-886-7742-9442; Email: [chemistry0220@gmail.com](mailto:chemistry0220@gmail.com)

\*\*\* University of Michigan, Ann Arbor, Michigan 48105, USA  
Telephone: (734) 623-9719; Email: [qiaoxiao@msu.edu](mailto:qiaoxiao@msu.edu)

**Abstract:** The real-time polymerase chain reaction (RT-PCR), also called quantitative real-time polymerase chain reaction (QRT-PCR) or kinetic polymerase chain reaction (kPCR), is a technique used to simultaneously quantify and amplify a DNA molecule. It is used to determine whether a specific DNA sequence is present in the sample; and if it is present, the number of copies in the sample. It is the real-time version of quantitative polymerase chain reaction (qPCR), itself a modification of polymerase chain reaction (PCR). 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 polymerase chain reaction to quantify messenger RNA (mRNA) at a particular time for in a particular cell or tissue type. [The Journal of American Science. 2006;2(3):1-15].

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### 1. Introduction

Polymease chain reaction (PCR) is a technique that allows logarithmic amplification of short DNA sequences (100 to 600 bases) within a longer double stranded DNA molecule. This technique was invented in 1980's by Dr. Kary Banks Mullis in California. Kary Banks Mullis awarded Norbel Prize of chemistry for the invention of PCR (Greer, 2006; Mullis, 2006). The purpose to put the three pictures of Dr. Mullis in this article is to see the characteriztion of the PCR inventor. From the photos we can see that Mullis is a thoughtful man. Let's thank and remember Dr. Mullis for the invetion of PCR when we do PCR.

Higuchi et al. pioneered the analysis of PCR kinetics by constructing a system that detects PCR products as they accumulate. This "real-time" system includes 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 cooled CCD camera. Amplification produces increasing amounts of double- stranded DNA, which binds ethidium bromide, resulting in an increase in fluorescence. By plotting the increase in fluorescence versus cycle number, the system produces amplification plots that provide a more complete picture of the PCR

process 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 (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 in the journal Biotechnology in 1993 as the following: "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).

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 followed the primer. After the new DNA synthesis, the same primers will be released and used again. This gives the DNA 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 the life science research is the discovery of a thermostable DNA polymerase (Taq polymerase) that is isolated from *Thermus aquaticus*, a bacterium that grows in hot pools near volcanic vent. 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 analyzed on an agarose gel and is abundant enough to be detected with an ethidium bromide (EB) stain. In order to measure messenger RNA (mRNA), the method is extended using reverse transcriptase to convert mRNA into complementary DNA (cDNA) which is amplified by PCR and, again analyzed by agarose gel electrophoresis. In many cases this method has been used to measure the levels of a particular mRNA under different conditions but the method is actually even less quantitative than PCR of DNA because of the extra reverse transcriptase step. Reverse transcriptase PCR analysis of mRNA is often referred to 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 anywhere the RT-PCR appears that represents real-time PCR.

There are two type quantifications from 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. When a particular protein is required by a cell, 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 and 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 it is still used to measure mRNA by

many laboratories with different proposes. Northern blot needs larger of mRNA sample, and RT-PCR was developed to measure small amount of mRNA. As the sensitivity if higher for RT-PCR method, the contamination could be pay attention. 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 at best because of the insensitivity of EB. PCR is the most sensitive method and can discriminate closely related mRNAs. Northern blot and ribonuclease protection assays are the standard methods, since no amplification is involved, whereas *in situ* hybridization is qualitative rather than quantitative. Techniques such as Northern blot and ribonuclease protection assays work very well, but require more RNA than is sometimes available. PCR methods are therefore particularly valuable when amounts of RNA are low, since the fact that PCR involves an amplification step means that it is more sensitive. In contrast to regular reverse transcriptase-PCR and analysis by agarose gels, RT-PCR gives quantitative results. An additional advantage of RT-PCR is the relative ease 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 study, 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).

In the traditional PCR technique, a PCR uses a peltier heat pump to quickly heat and cool the DNA and uses the Taq polymerase. *Thermophilus aquaticus* (Taq) is a bacterium that lives by volcanic sulfer jets at the bottom of the ocean. They can withstand extreme temperatures, and that is why they are so valuable in PCR. Primers are short strands of RNA that bind to specific known sites on the DNA molecule. DNA polymerases need to have RNA primers in order to begin replication. Four dNTPs (deoxyribonucleotide triphosphates) (dGTP, dCTP, dATP and dTTP) are letters of the DNA alphabet and the taq polymerase uses the sNTPs to build the new molecular chains.

#### **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.

PCR makes 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).

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 using either labeled hybridization probe(s) (Taq Man, Molecular Beacons) or labeled PCR primer (Amplifluor) and SYBR Green (Applied Biosystems).

## 2. 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 cycle threshold ( $C_T$ ) 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 contal 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 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. 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  $\Delta C_T$ , 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 analysed. 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 moiety (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 end-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.

### **1) 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 moiety 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).

### **2) 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.

### **3) 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.

### **4) 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.

## 5) 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.

## 6) 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<sup>32</sup>-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.

### (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 — 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 β-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 β-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 competitors — primers identical in sequence to the functional 18S rRNA primers but that are blocked at their 3' end and cannot be extended by PCR. Competitors 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 competitors 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  $\beta$ -actin as an appropriate internal control for their system, the QuantumRNA  $\beta$ -actin Internal Standards are available.

### (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. 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.

### (3) 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.

### 3. Brief Description for the RT-PCR Procedure (Protocol online, 2006)

- 1) 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.
- 2) cDNA is denatured at more than 90°C (~94°C) so that the two strands separate. The sample is cooled to 50°C to 60°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.
- 3) The temperature is raised to 72°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°C.
- 4) Again, the primers are annealed at a suitable temperature (normally between 50°C and 60°C).
- 5) Taq DNA polymerase binds and extends from the primer to the end of the cDNA strand. There are now eight cDNA strands
- 6) Again, the strands are denatured by raising the temperature to 94°C and then the primers are annealed at 60°C.
- 7) The temperature is raised and the polymerase copies the eight strands to sixteen strands.
- 8) The strands are denatured and primers are annealed.
- 9) The fourth cycle results in 32 strands.
- 10) 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 intercalation (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)

#### 4. Time Required for RT-PCR

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 1).

Table 1. Time required for RT-PCR

1	cDNA synthesis: 2 hours
2	RT-PCR: 2 hours
3	Dissociation curve analysis: 0.5 hour
Total Time	4.5 hours

#### 5. 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 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.

##### 2) Comparative C<sub>T</sub> Method

As described earlier, C<sub>T</sub> is the cycle threshold. Another quantitation method for RT-PCR described

here is the comparative  $C_T$  method. 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  $2^{-\Delta\Delta C_T}$  method, where  $\Delta\Delta C_T = \Delta C_{T,\text{sample}} - \Delta C_{T,\text{reference}}$ . Here,  $\Delta C_{T,\text{sample}}$  is the  $C_T$  value for any sample normalized to the endogenous housekeeping gene and  $\Delta C_{T,\text{reference}}$  is the  $C_T$  value for the calibrator also 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  $\Delta C_T$  varies with template dilution. If the plot of cDNA dilution versus  $\Delta C_T$  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.

## 6. RT-PCR Equipments and Reagents

### 1) Instruments for RT-PCR

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 & 0.2 ml Tube Instruments:

**A. 7000 Real-Time PCR System:** The ABI Prism® 7000 Sequence Detection System effective

Summarily, the current prices of the RT-PCR instruments by Applied Biosystems are as the following:

The Applied Biosystems 7300 Real-Time PCR System                   US\$ 34,900.00

The Applied Biosystems 7500 Real-Time PCR System                   US\$ 42,500.00

The Applied Biosystems 7500 Fast Real-Time PCR System           US\$ 49,900.00

7900HT Fast Real-Time PCR System                                   US\$ 131,500.00

(Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, USA. Telephones: 650-638-5800, 1800-327-3002;

March 31, 2006. After this date, the ABI Prism® 7000 Sequence Detection System will no longer be available for sale. We have decided to discontinue the sale of this product because of the introduction of newer and more affordable technology.

**B. 7300 Real-Time PCR System:** The Applied Biosystems 7300 Real-Time 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\$34,900.00.

**(2) 7500 Real-Time PCR System:** The Applied Biosystems 7500 Real-Time PCR System is a leading edge system with features to support labs requiring more capabilities from their real time PCR platform. The current price is US\$42,500.00.

#### (3) 96-Well Instruments

**7500 Fast Real-Time PCR System:** The Applied Biosystems 7500 Fast Real-Time 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\$49,900.00.

#### (4) 96- & 384-Well Instruments

**7900HT Fast Real-Time PCR System:** The Applied Biosystems 7900HT Fast Real-Time PCR System is the only real-time quantitative 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 real-time PCR capability. The current price is US\$131,500.00.

Fax: 650-638-5884, Email: [NA\\_ActSrvInfo@appliedbiosystems.com](mailto:NA_ActSrvInfo@appliedbiosystems.com), Website: <http://www.appliedbiosystems.com>.

## 2) Reagents and Tools for RT-PCR

Table 2 gives the basic reagents and equipments needed for RT-PCR of SYBR method (Table 2).

Table 2. Reagents and equipments needed 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 3 gives the basic reagents and equipments needed for RT-PCR of TaqMan method (Table 3).

Table 3. Reagents and equipments needed 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 real-time 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 scientists 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. Cells-to-cDNA II is compatible with both one-step and two-step real-time RT-PCR protocols.

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 or better than the more expensive alternatives, and includes dNTPs and a Reaction Buffer optimized for SYBR Green, TaqMan, and Molecular

### 7. Detailed Procedure of RT-PCR

In a 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: Non-Specific detection using DNA binding dyes and specific detection target specific probes (PREMIER Biosoft International., 2006).

### **1) Non-specific 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 real-time 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 strand 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.

### **2) 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

### **3) 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 real-time methods. 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 (Table 4).
- c. TaqMan® EZ RT-PCR Core Reagents combine rTth DNA polymerase with the fluorogenic 5' nuclease assay in a one-step reverse transcription-PCR (RT-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 5).
- d. The TaqMan® Gold RT-PCR kit provides the individual components needed to perform reverse transcription-PCR (RT-PCR) of RNA to cDNA using the 5' nuclease assay. You can use this kit to perform both one-step and two-step RT-PCR (Table 6).

**B. Two-step reagent:** Requires two reaction mixes (real-time reaction and PCR reaction) and cDNA can be store for later use. Using random primer, it can simultaneously reversely transcribe all mRNA as well as 18s rRNA (targets+endogenous controls), and it can use

sequence-specific primer, random primer or oligo d(T)<sub>16</sub> for cDNA synthesis.

Table 4. 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	590.00
10-Pack, TaqMan® One-Step RT-PCR Master Mix Reagents Kit	4313803	2000 reactions	5420.00

Table 5. 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	755.00
10-Pack, TaqMan® EZ RT-PCR Core Reagents	403028	2000 reactions	6900.00

Table 6. 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	755.00
10-Pack, TaqMan® Gold RT-PCR Reagents without	4304133	2000 reactions	6900.00

controls			
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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 (USA) and other companies also have complete kit for RT-PCR.

- A. Prepare RNA/primer mixture in each tube (Table 7).
- 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 8)
- 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 1st strand cDNA at -20°C until use for RT-PCR.

Table 7. RNA/primer mixture

Total RNA	5 µg
Random hexamers (50 ng/µl)	3 µl
10 mM dNTP mix	1 µl
DEPC H <sub>2</sub> O	to 10 µl

Table 8. Reaction master mixture

10x RT buffer	2 µl
25 mM MgCl <sub>2</sub>	4 µl
0.1 M DTT	2 µl
RNAaseOUT	1 µl

#### 4) RT-PCR Procedure

- A. Normalize the 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 9).
- 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 9. RT-PCR reaction mixture of 50 µl or 25 µl

25 µl SYBR Green Mix (2x)	Or	12.5 µl SYBR Green Mix (2x)
0.5 µl tissue cDNA		0.25 µl tissue cDNA
2 µl primer pair mix (5 pmol/µl each primer)		1 µl primer pair mix (5 pmol/µl each primer)
22.5 µl H <sub>2</sub> O		11.3 µl H <sub>2</sub> O

## 7. Applications of RT-PCR

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 EB 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 real-time PCR are growing exponentially, real-time 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. Real-time 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 real-time 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, real-time 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 has 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-PCR will be very benefit on the gene expression studies (Ma, 2006).

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

1. Quantitative gene expression studies (mRNA synthesis) (qPCR).
2. DNA copy number measurements in genomic or viral DNAs.
3. Allelic discrimination assays or SNP genotyping.
4. Microarray result verifications.
5. Drug designs.
5. Drug therapy efficacy exploring.
6. DNA damage measurements.

## 8. Troubleshooting

- 1) **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.
- 2) **Poor PCR amplification efficiency.** The accuracy of real-time 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.

- 3) **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 limit 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 prime formation.
- 4) **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 melting temperature 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 fuzzy) 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.
- 5) **Non-specific amplicons.** Non-specific amplicons, identified by both gel electrophoresis and melting curve analysis, give misleading real-time 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).

## 9. Biography of Kary Banks Mullis

Lastly, in the following it gives the PCR inventor Kary Banks Mullis' biography:

- 1) 1944, December 28: Kary Banks Mullis (male) was born on in Lenoir North, North Carolina, USA.
- 2) 1966: received a bachelor degree in chemistry from the Georgia Institute of Technology, USA.
- 3) 1972: obtained his Ph.D. in biochemistry from the Berkeley of University of California, USA.
- 4) 1972 - 1973: Taught biochemistry at Berkeley Berkeley of University of California, USA.
- 5) 1973 – 1977: postdoctoral fellow in pediatric cardiology (in the areas of angiotensin and pulmonary vascular physiology) at the University of Kansas Medical School, USA.
- 6) 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.**
- 7) 1986: director of molecular biology at Xytronyx, Inc. in San Diego of California of USA, on DNA technology and photochemistry.
- 8) 1987: began consulting on nucleic acid chemistry for more than a dozen corporations, including Angenics, Cytometrics, Eastman Kodak, Abbott Labs, Milligen/Bioscience and Specialty Laboratories.
- 9) 1990: awarded the William Allan Memorial Award of the American Society of Human Genetics, USA.
- 10) 1990: awarded the Award of Preis Biochemische Analytik of the German Society of Clinical Chemistry and Boehringer Mannheim, German.
- 11) 1991: awarded the National Biotechnology Award, USA.
- 12) 1991: awarded the Gairdner Award, Toronto, Canada.
- 13) 1991: awarded the R&D Scientist of the Year, USA.
- 14) 1992: awarded California Scientist of the Year Award, USA.
- 15) 1993: obtained a Nobel Prize in chemistry for his invention of PCR.**
- 16) 1993: obtained the Japan Prize for the PCR invention, Japan. It is one of international science's most prestigious awards.
- 17) 1993: obtained Thomas A. Edison Award, USA.
- 18) 1994: obtained the honorary degree of Doctor of Science from the University of South Carolina, USA.
- 19) 1998: inducted into the National Inventors Hall of Fame, USA.
- 20) 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.
- 21) 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.
- 22) 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.
- 23) 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).
- 24) Dr. Kary Banks Mullis' website is: <http://www.karymullis.com>.

### Correspondence to:

Hongbao Ma  
Michigan State University  
East Lansing, MI 48823, USA  
Telephone: (517) 303-3990  
Email: [hongbao@msu.edu](mailto:hongbao@msu.edu); [hongbao@gmail.com](mailto:hongbao@gmail.com)

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