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Real-Time PCR for Gene Expression Analysis

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1. Introduction

After discovery of polymerase chain reaction (PCR) by Dr. Kary Mullis in 1983, several different types of PCR have been invented and continually improved upon over the years. One of them called "Real-time PCR" or "fluorescence based PCR" allows us to quantitate nucleic acids obtained from cells or tissues, to compare the variable states of infection, to detect chromosomal translocations, to genotype single nucleotide polymorphisms, to determine gene expression level of samples and so on. For the detection and quantification of nucleic acids, Real-time PCR has become the most accurate and sensitive method. Quantitative measurement of specific gene expression using quantitative PCR (qPCR) is necessary for understanding basic cellular mechanisms and detecting of alteration in gene expression levels in response to specific biological stimuli (e.g., growth factor or pharmacological agent) (Bustin, 2000; Bustin, 2002). Quantification of nucleic acids has been significantly simplified by the development of the Real-time PCR technique (Bustin, 2002; Huggett et al., 2005). It is mostly used for two reasons: either as a primary investigative tool to determine gene expression or as a secondary tool to validate the results of DNA microarrays (Valasek & Repa, 2005).

2. Basic principles of PCR and Real-time PCR

PCR is an easy and quick *in vitro* method to amplify any target DNA fragment (Powledge, 2004). In PCR, DNA polymerase enzymes are used for the amplification of specific DNA fragments. For this purpose, the most commonly used DNA polymerase is called *Taq* DNA polymerase, isolated from thermophilic bacteria, *Thermus aquaticus*. Another enzyme named *Pfu* DNA polymerase, isolated from *Pyrococcus furiosus*, is also used for PCR because of its higher fidelity during amplification of DNA. These two enzymes are heat stable and DNA dependent DNA polymerases. They can synthesize new DNA strand using a DNA template in the presence of primers, deoxyribonucleotide triphosphates (dNTPs), Mg^{2+} and proper buffer system (Old & Primrose, 1994; Valasek & Repa, 2005).

The PCR involves two oligonucleotide primers which flank the DNA sequence that is to be amplified. The primers hybridize to opposite strands of the DNA after it has been denatured, and are oriented so that DNA synthesis by the polymerase proceeds through the region between the two primers. PCR involves three steps: denaturation, primer hybridization or annealing and extension. During the extension, polymerase creates two

double stranded target regions, each of which can again be denatured ready for a second cycle of hybridization and extension (Fig. 1A). The third cycle produces two double-stranded molecules that comprise precisely the target region in double-stranded form. As shown in Fig. 1B, by repeated cycles of heat denaturation, primer hybridization and extension, there follows a rapid exponential accumulation of specific target fragment of DNA (Old & Primrose, 1994).

If the reaction runs with perfect efficiency (100%), there will be two fold increases in target DNA fragment after each cycle of PCR. For example; after n cycles of PCR, the copy number of the target fragment will be 2^n . In practice, reactions, however, do not work with perfect efficiency as reactants within PCR mixture are depleted after many cycles, and then the reaction will reach a plateau phase in which no change of the amount of the product (Gibson et al., 1996; Heid et al., 1996; Valasek & Repa, 2005).

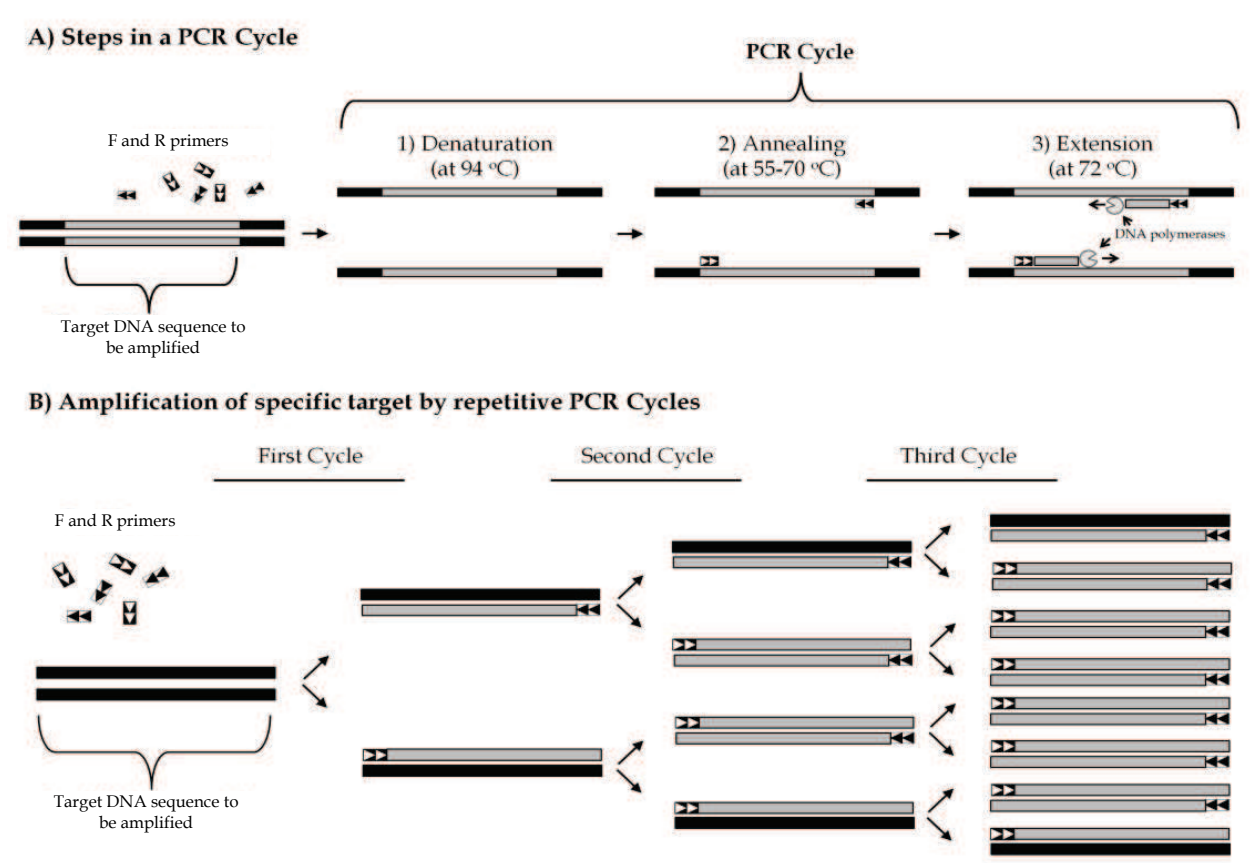


Fig. 1. Schematic representation of PCR. A: Steps in a PCR cycle.
B: Exponential amplification of specific target by repetitive PCR cycles.

PCR can be divided into four phases: the linear ground phase, exponential phase, log-linear phase and plateau phase (Fig. 2) (Tichopad et al. 2003). The advantage of using fluorogenic dyes in the Real-time PCR experiments is to visualize these phases during the reaction. Real-time PCR exploits the fact that the quantity of PCR products in exponential phase is in proportion to the quantity of initial template under ideal conditions (Gibson et al., 1996; Heid et al., 1996).

At the linear ground phase, (usually the first 10-15 cycles), fluorescence emission produced at each cycle has not been higher than the background. Thus, it is obscured by the background fluorescence. This fluorescence is calculated at linear ground phase (Tichopad et al., 2003).

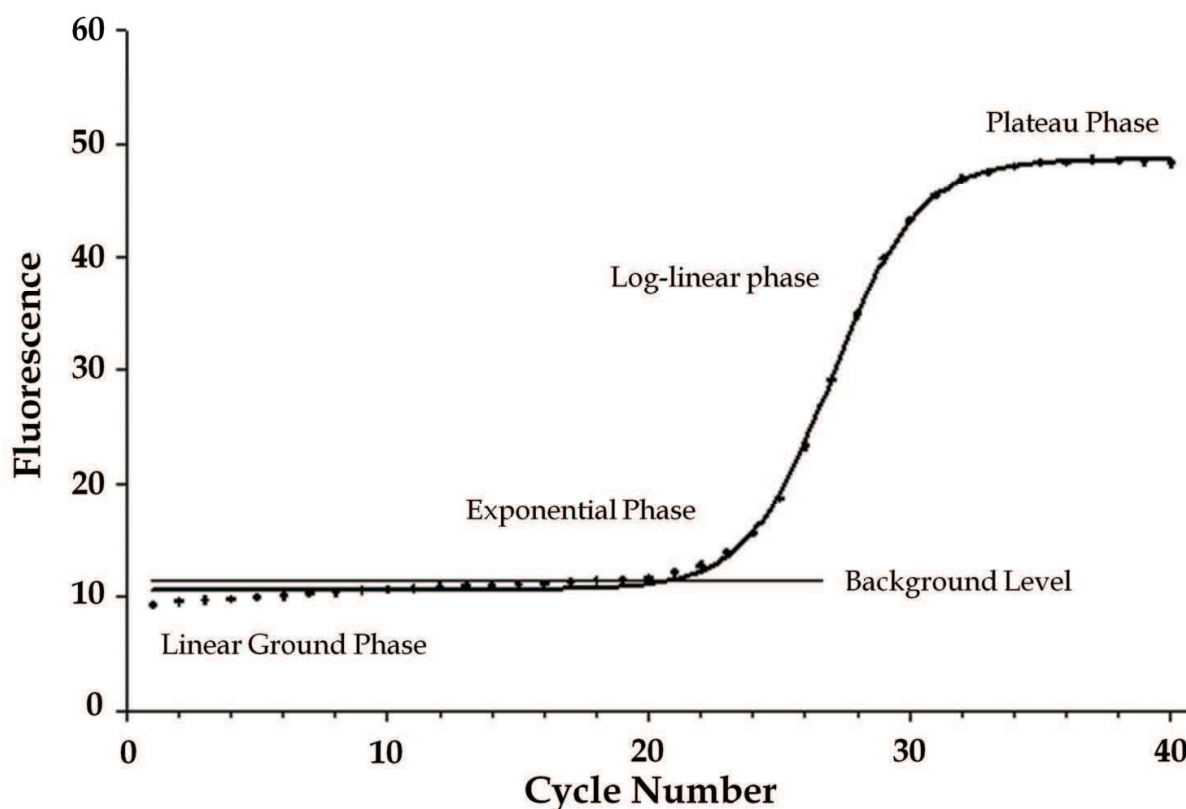


Fig. 2. The four phases of PCR shown in a plot of fluorescence signal versus cycle number.

At exponential phase, the amount of fluorescence reaches a threshold where it can be detected as significantly stronger than the background fluorescence signal. The cycle in which this detection happened is known as threshold cycle (Ct) in ABI PRISM® literature (Applied Biosystems, Foster City, USA) or crossing point (Cp) in LightCycler® literature (Roche Applied Science, Indianapolis, USA) (Tichopad et al. 2003). This value will be called as Ct throughout the text. Ct is a very important point of Real-time PCR because this value represents the amount of target gene found in the sample and it is used to calculate experimental results. If the amount of target sequence is high in the sample, the reaction reaches exponential phase more quickly and thus, the cycle (or Ct value) in which the amount of fluorescence reaches a threshold will be lower for this sample (Heid et al., 1996).

In log-linear phase, the reaction takes place with linear efficiency and increase in fluorescence signal in every cycle is occurred. As stated before, in the last phase of reaction, plateau phase, reactants become limited and exponential product accumulation do not occur anymore (Gibson et al., 1996; Heid et al., 1996; Tichopad et al. 2003).

It is important to underlie that the quantity of PCR products in exponential phase correlates to the quantity of initial template under an ideal conditions (Gibson et al., 1996; Heid et al., 1996). Because the reaction efficiently accomplishes DNA amplification only up to a certain quantity before the plateau effect, it is not possible to reliably calculate the amount of starting DNA by quantifying the amount of product at the end of reaction. After reaction, similar amounts of amplified DNA in the samples that contain different amount of a specific target DNA sequence is found because of plateau effect. Thus, any distinct correlation between samples is lost. Real-time PCR solves this problem by measuring product formation during exponential phase since efficient amplifications occur early in the reaction process (Valasek & Repa, 2005).

3. Template preparation

The first step in gene expression studies is isolation of the high quality RNA from samples. RNA is chemically less stable than DNA so that maintaining RNA integrity in an aqueous solution and protection of RNA against degradation is very important (Fraga et al., 2008). There are numerous protocols and commercially available kits for isolating total RNA and/or mRNA from different tissue samples and some of them are tissue specific (Bustin, 2002; Fraga et al., 2008).

Inhibitory components present frequently in biological samples may cause a significant reduction in the sensitivity and kinetics of Real-time PCR (Radstrom et al., 2004). These inhibitors may originate from reagents used during nucleic acid extraction or co-purified components from the biological sample, for example bile salts, urea, heme, heparin or IgG (Nolan et al., 2006). The presence of any inhibitors of polymerase activity in both reverse transcription and Real-time PCR steps should be considered crucially as many biological samples contain inhibitors for the polymerases (Smith et al., 2003). Inhibitors affect the experiment in two ways by generating incorrect quantitative results or creating false-negative results. The presence of inhibitors within biological samples can be checked by various methods (Nolan et al., 2006).

Differences in mRNA expression patterns at the cellular level may also be masked because of by variability resulting from RNA samples extracted from complex tissue specimens. Such tissues contain variable subpopulations of cells of different lineage at different stages of differentiation. Moreover, malign tissue specimens may also consist of normal cells such as epithelial, stromal, immune or vascular cells. Thus, Real-time PCR data obtained from such a mixed sample is an average of different cell populations. To solve this problem, cell sorting technique can be used for enriching specific cell populations using flow cytometry or antibody-coated beads for blood samples (Deggerdal & Larsen 1997; Raaijmakers et al., 2002;). However, there is no practical way of sorting cells without affecting the expression profile of the sample for solid tissue biopsies. This variability may be partly excluded after tumor and normal tissue samples checked by pathologist view before starting Real-time PCR experiment. Moreover, the introduction of laser capture microdissection (LCM) technique promises to address this particular problem. By using this technique, target mRNA levels can be reported conveniently as copies per area or cells dissected (Fink et al., 1998).

Furthermore, total RNA extracted tissue specimens are usually contaminated with DNA. If the tissue has high DNA content, DNase I treatment is necessary to eliminate residual DNA. If the samples are to be DNase-treated, it is compulsory to remove DNase before cDNA synthesis (Bustin, 2002). After isolation, RNA should be stored at -80°C.

Traditionally, the ratio of absorbance at 260 nm and 280 nm or analysis of the rRNA bands on agarose gels are used to determine the purity of RNA. OD 260/280 ratio higher than 1.8 is accepted as proper for downstream applications (Manchester, 1996). RNA is considered of high quality when the ratio of 28S:18S bands is about 2.0. Nowadays, Agilent BioAnalyser, Ribogreen, NanoDrop and BioRad Experion are used for this purpose (Nolan et al., 2006). NanoDrop ND-1000 spectrophotometer only needs 1 µl of sample and can be used with concentrations as low as 2 µg ml⁻¹.

Agilent 2100 Bioanalyzer and BioRad Experion are used for the quality control of RNA. These instruments use a lab on a chip approach to perform capillary electrophoresis (Nolan et al., 2006). These instrument softwares calculate a numerical value: RNA integrity number (RIN) on the 2100 Bioanalyzer and quality index (RQI) on the Experion. A RQI/RIN of 1 exhibits nearly fragmented and degraded RNA and a RQI/RIN of 10 exhibits intact and non-degraded RNA (Schroeder et al., 2006). RNA quality score (RIN or RQI) higher than five is determined as good total RNA quality, moreover, higher than eight is perfect total RNA for gene expression studies (Fleige & Pfaffl, 2006).

After isolation of total RNA and evaluating its integrity and purity, cDNA synthesis can be simply made using commercially available kits starting with equal amounts of RNA samples. Moreover, cDNA can be synthesized using random primers, oligo(dT), target gene specific primers or a combination of oligo(dT) and random primers.

4. Real- time PCR primer design

Optimal primers are essential to insure that only a single PCR product is amplified. In order to avoid non-specific PCR products, primers should not have high sequence similarity with other sequences. This can be checked using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Primers containing 16-28 nucleotides are enough for successful PCR amplification. GC content of the primer should be between 35%-65% (Wang & Seed, 2006).

In addition to general rules used for designing common PCR primers, some important parameters should be considered for Real-time PCR amplification. Primers should be designed to give product size of 100-200 bp. Primer melting temperatures (T_m) should be 60–65 °C. Intron spanning primer pair should be preferred in order to prevent potential signals from genomic DNA contamination in the sample. Finally, if oligo(dT) is used for priming in reverse transcription, primers should be located within 1000 bp of the 3' end of mRNA (Wang et al., 2006).

There are some free online tools or commercially available softwares which can be used for primer design if the parameters described above are provided. The selected list of useful web resources and some commercial programs is given in table 1.

Website or Software Name	Specification	URL
Primer3	Picking primer and hybridization probes	http://frodo.wi.mit.edu/
Primer-BLAST	For making primers. It uses Primer3 to design primers and then submits them to BLAST search	http://www.ncbi.nlm.nih.gov/
PrimerBank	Public database of Real-time PCR primers. Contains over 306.800 primer mostly for human and mouse	http://pga.mgh.harvard.edu/
RTPrimer DB	Public database for primer and probe sequences used in real-time PCR assays. Contains 8309 real-time PCR assays for 5740 genes.	http://www.rtprimerdb.com/
Real-time PCR Primer Sets	Primer and Probe database, mostly for SYBR green assays	http://www.realtimeprimers.com/
OligoCalc	Calculates oligonucleotide properties	http://www.basic.northwestern.edu/
Universal Probe Library from Roche Applied Science	Designing primers and UPL hydrolysis probe	www.universalprobelibrary.com http://www.roche-applied-science.com/
Primer Express	Designing primers and TaqMan probes	www.appliedbiosystems.com
Beacon Designer	Real-time PCR primers and probes	http://www.premierbioscience.com/
Primer Premier	Primer Design	http://www.premierbioscience.com/

Table 1. Some free online tools or commercially available softwares

5. Real-time PCR detection chemistries

In the following section we will focus on the detection chemistries which deviates Real-time PCR from conventional PCR assays. Real-time PCR detection chemistries can be classified into sequence non-specific or specific detection chemistries.

5.1 Sequence non-specific detection

The principle of the sequence non-specific detection is the use of DNA binding fluorogenic dyes. This method is not affected when the presence of variations (i.e. single nucleotide polymorphisms or SNPs) on the target sequence. Moreover, less specialist knowledge is required as compared to the design of fluorogenic oligoprobes (Komurian-Pradel et al., 2001). DNA binding dyes are also inexpensive and simple to use (VanGuilder et al., 2008).

The first dye used as DNA binding fluorophore was ethidium bromide (Higuchi et al., 1993; Wittwer et al., 1997), and other dyes such as SYBR Green I, YO-PRO and BEBO have been also used (Ishiguro et al., 1995; Tseng et al., 1997; Morrison et al., 1998; Bengtsson et al., 2003). All these dyes fluoresce when binding with double-stranded DNA (dsDNA) and this dsDNA-dye complex is revealed by a suitable wavelength of light. Thus, observing the amplification of any dsDNA template is possible during reaction (Fig. 3).

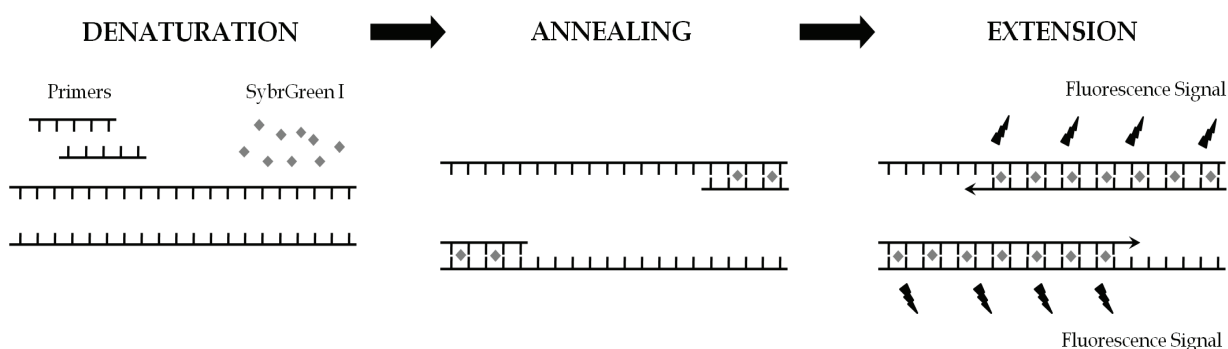


Fig. 3. Detection principle of SYBRGreen I dye during PCR amplification.

SYBR Green I is the most frequently used dsDNA specific dye in Real-time PCR. It is a cyanine dye with an asymmetric structure. The binding affinity of SYBR Green I to dsDNA is 100 times higher than that of ethidium bromide (Wittwer et al., 1997; Morrison et al., 1998). After SYBR Green I binds to dsDNA, it emits 1000-fold greater fluorescence as compared to unbound dyes (Wittwer et al., 1997).

As the amount of double-stranded amplification product increases during reaction, the amount of dye that can bind and fluoresce, also increases. Thus, the fluorescence signal elevates proportionally to dsDNA concentration (Wittwer et al., 1997). However, these dyes also bind primer-dimers, commonly occur during reaction, and non-specific PCR products. This non-specific dsDNA-dye interaction can cause misinterpretation of the results. That is why these dyes provide sensitive but not specific detection (Espy et al., 2006). However, this problem can be solved using melting curve analysis. Instruments performing a melting curve analysis to determine the T_m allow detection of accumulation of different products based upon the G+C% content and length of the amplification product (Espy et al., 2006).

After melting curve analysis, if two or more peaks are present, it means that there are more than one amplified products in the reaction and thus no specific amplification for a single DNA sequence is occurred (Valasek & Repa, 2005).

5.2 Sequence specific detection

Development of fluorescent probe technology allows us to perform sensitive and specific detection with Real-time PCR. Mainly, there are three types of probes used in the reaction although they have distinct molecular structure and dyes attached. These probes can be grouped as follows: hybridization probes, hydrolysis probes and hairpin probes. All detection methods using fluorescent probe technology rely on a process referred to as fluorescence resonance energy transfer (FRET) in which the transfer of light energy between two adjacent dye molecules occurs (Espy et al., 2006). However, both hydrolysis and hybridization probes depend on FRET to change fluorescence emission intensity, the energy transfer works in opposite manners in these two chemistries. While FRET reduces fluorescence intensity in hydrolysis probes, it increases intensity in hybridization probes (Wong & Medrano, 2005).

5.2.1 Hybridization probes

One or two hybridization probes can be used in a reaction (Bernard & Wittwer, 2000). In an assay utilizing two hybridization probes, they bind to target sequence in close proximity to each other in a head-to-tail arrangement (Wittwer et al., 1997a; Wong & Medrano, 2005;). The upstream probe carries an acceptor (or quencher) dye on its 3' end the second probe or downstream probe is labeled with a donor (or reporter) dye on 5' end (Wittwer et al., 1997; Bustin, 2000; Wong & Medrano, 2005). On the other hand, in one probe method, the upstream primer is labeled with an acceptor dye on the 3' end instead of labeling probe. Thus, labeled primer replaces the function of one of the probes used two hybridization probe method (Wong & Medrano, 2005). In both cases, the energy transfer depends on the distance between two dye molecules. Because of the distance between two dyes in solution, donor dye emits only background fluorescence (Bustin, 2000). When the probes hybridize to their complementary sequence, this binding brings the two dyes in close proximity to one another and FRET occurs at high efficiency. Since, a fluorescent signal is detected only as a result of two independent probes hybridizing to their correct target sequence, increasing amounts of measured fluorescence is proportional to the amount of DNA synthesized during the PCR reaction. Moreover, as the probes are not hydrolyzed, fluorescence signal is reversible and allows the generation of melting curves (Bustin, 2000) (Fig. 4).

5.2.2 Hydrolysis probes

Hydrolysis probes (also known as TaqMan probes or 5' nuclease assay) contain a fluorescent reporter dye at its 5' end and quencher dye at its 3' end (Wong & Medrano, 2005; VanGuilder et al., 2008). If the probe is unbound, reporter and quencher dyes are maintained in close proximity, which allows the quencher to reduce the reporter fluorescence intensity by FRET, and thus no reporter fluorescence is detected (Bustin, 2000) (Fig. 4).

On the other hand, the probe binds to the target sequence, when the specific PCR product is generated. It remains hybridized while the polymerase extends the primer until the enzyme reaches the hybridized probe. Then the 5'-3' exonuclease activity of DNA polymerase degrades the probe during extension step of the PCR (Heid et al., 1996). 5'- exonuclease activity of the polymerase releases the 5' reporter dye from the quenching effect of the 3' dye and this release is detected as an increase in fluorescence intensity (Heid et al. 1996; Bustin, 2000; VanGuilder et al., 2008) (Fig. 4).

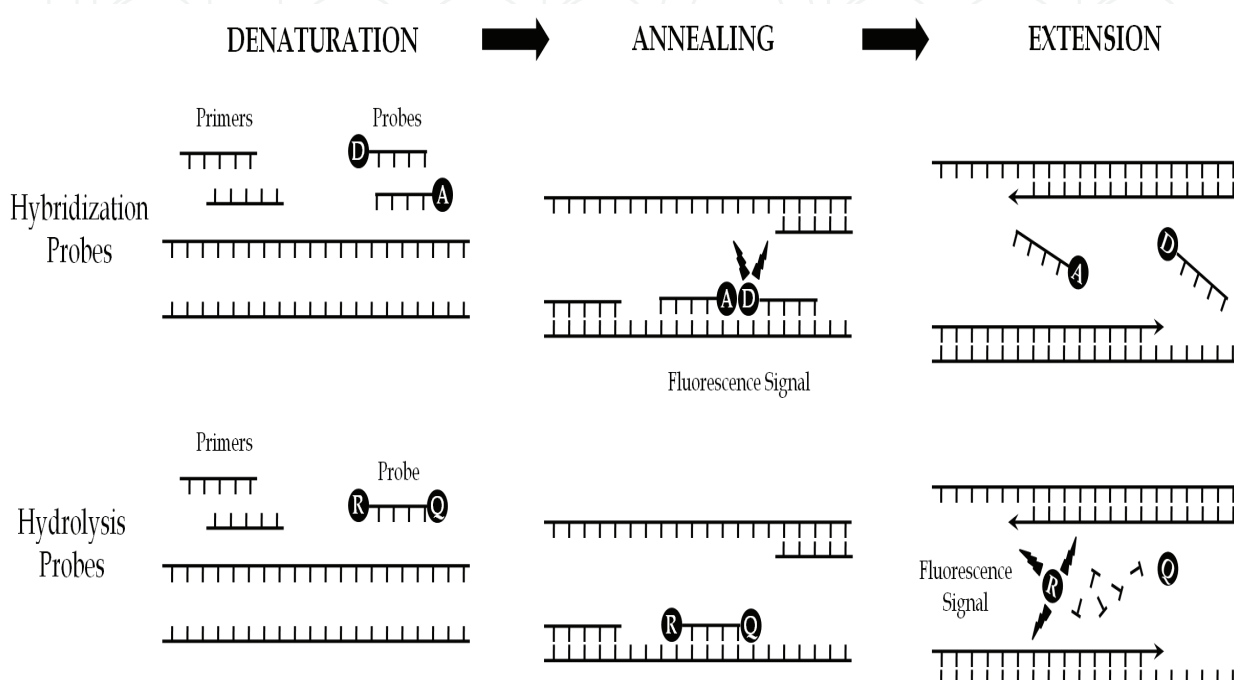


Fig. 4. Detection of nucleic acids using hybridization and hydrolysis probes in Real-time PCR.

Hydrolysis probes commonly are in structure of nucleic acids, however, recently developed, Locked Nucleic Acids (LNA) containing hydrolysis probes are commercially available from Roche Applied Science under the name of Universal Probe Library (UPL) probes and can be accessed online from the site given in Table 1. LNAs are DNA nucleotide analogues with increased binding strengths compared to standard DNA nucleotides. In order to maintain the specificity and T_m , LNA bases are incorporated in each UPL probes (www.universalprobelibrary.com).

5.2.3 Hairpin probes

When compared with linear DNA probes, hairpin or stem-loop DNA probes have an increased specificity of target recognition. Hairpin DNA probes are single-stranded oligonucleotides and contain a sequence complementary to the target that is flanked by self-complementary target unrelated termini. Invention of hairpin probes is let to view hybridization process in real-time. They are widely used in different applications and two major factors are responsible for such broad applications of these DNA probes: Enhanced specificity of the probe-target interaction and the possibility of closed-tube real-time monitoring formats (Broude, 2005). There are several types of hairpin probes commercially

available including molecular beacons, scorpions, LUXTM fluorogenic primers and SunriseTM Primers.

5.2.3.1 Molecular beacons

Molecular beacons are a class of hairpin probes and first developed in 1996 (Tyagi & Kramer 1996). Sequence-specific loop region is located between two inverted repeats which form stem of the hairpin by complementary base pairing (Tyagi & Kramer 1996; Bonnet et al., 1999). Reporter and quencher dyes are linked to each end of the molecular beacon. In solution, reporter's fluorescence is effectively reduced via contact quenching. When probe binds to the target sequence, reporter and quencher dyes separates, resulting in increased fluorescence (Tan et al., 2000) (Fig. 5A). The fluorescence of the probe increases 100-fold when it binds to its target (Bonnet et al., 1999).

5.2.3.2 Scorpions

Scorpion primers are bi-functional molecules because probe sequence is covalently linked to primer. Probe sequence forms stem-loop structure and contains fluorophore at 5'-end which is quenched by a moiety attached to the 3'-end of the loop. The probe is linked to the 5'-end of a primer via a nonamplifiable monomer or DNA polymerase blocker (Whitcombe et al., 1999). The probe part of the scorpion is complementary to the extension product of the attached primer. After extension step in a PCR, the probe will bind to the extended part of the primer when the complementary strands are separated in the denaturation step of the next cycle. Therefore, scorpion primers generate self-probing PCR products (Whitcombe et al., 1999; Thelwell et al., 2000) (Fig. 5B).

5.2.3.3 LUXTM fluorogenic primers

Light Upon eXtension (LUX) primers (Invitrogen, Carlsbad, CA, USA) are self-quenched single-fluorophore labeled primers. It is designed to be self-quenched with secondary structure of the its 3' end (Nazarenko et al., 2002a). This secondary structure reduces initial fluorescence to a minimal amount until the primer is incorporated into a double-stranded PCR product (Nazarenko et al., 2002b). This incorporation causes an unfolding of the primer which abolishes the self-quenching and thus an increase in fluorescence occurs (Kusser, 2006) (Fig. 5C). LUX primers are designed to have a G or C 3'-terminal nucleotide and fluorophore attached to the second or third base (Thymine nucleotide) from the 3' end. It also has five to seven nucleotide 5'-tail that is complementary to the 3' end of the primer. Such a design of the primer allows the molecule to form a blunt-end hairpin structure with low fluorescence at temperatures below its T_m . Various fluorescent dyes can be used, allowing the potential for multiplex assays to simultaneously quantitate multiple genes (Nazarenko et al., 2002b).

5.2.3.4 SunriseTM primers

Sunrise primers consist of a dual-labeled (reporter and quencher) hairpin loop on the 5' end. Similar to the scorpions, their 3' end is used as a PCR primer by the polymerase (Nazarenko et al., 1997). However, the probe part of the Scorpion is complementary to the extension product of the attached primer. On the other hand, the probe sequence of the Sunrise primers is complementary to the hybridized strand of the primer. After integration of the Sunrise primer into the newly formed PCR product, the reporter and quencher locate far

enough which allow reporter emission (Wong & Medrano, 2005) (Fig. 5D). It is important to consider that Sunrise primers may produce fluorescence signals due to nonspecific products and primer-dimers.

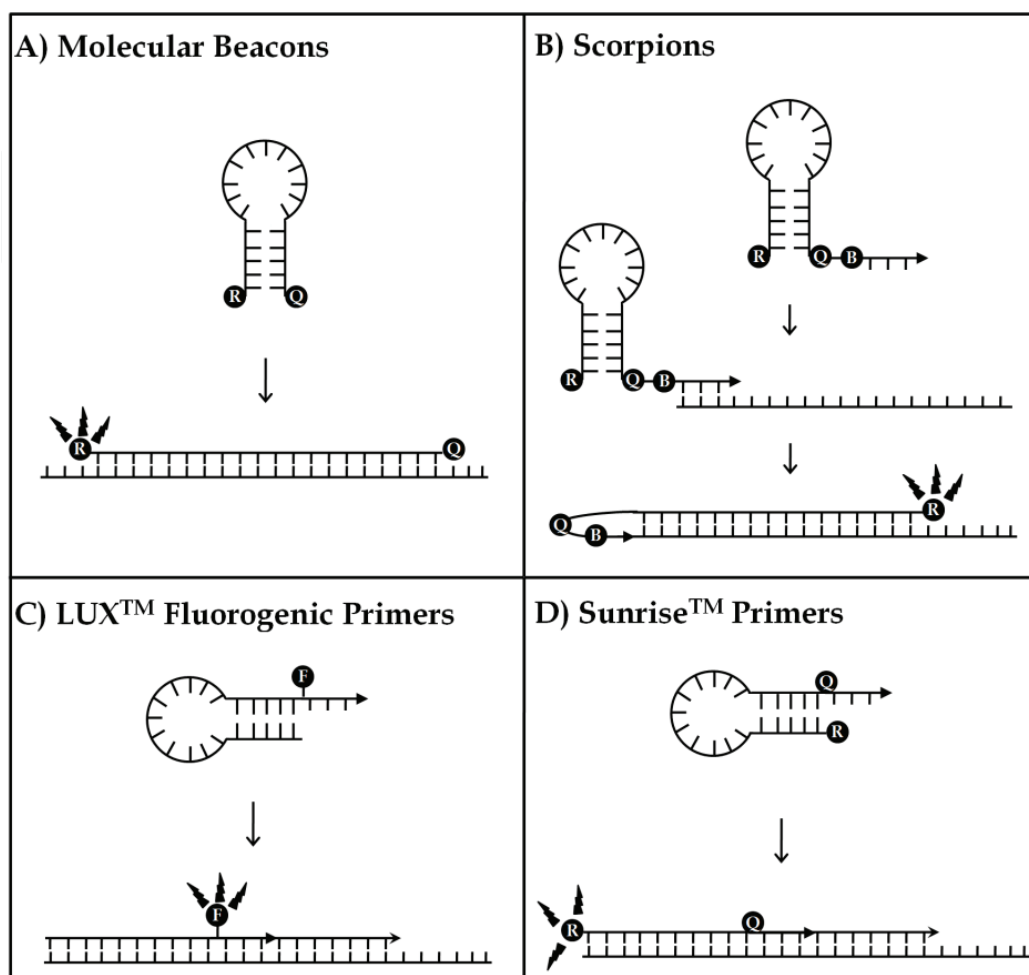


Fig. 5. Representative diagram showing hairpin probes and their principles of detection. A: Molecular beacons. B: Scorpions. C: LUX™ fluorogenic primers and D: Sunrise™ primers.

5.2.3.5 Other detection chemistries

Recently, newly developed detection chemistries, which will not be discussed in further detail here, have been introduced for Real-time PCR. Like hybridization and hydrolysis probes, these new systems all rely on the FRET principle. Although the list of these new chemistries is rapidly growing, some of them are minor groove-binding probes (MGB probes), ResonSense probes, Hy-beacon probes, Light-up probes, Simple probes, Lion probes, AllGlo probes, Displacement probes and etc (Overbergh et al., 2010). Some of these probes contain synthetic nucleic acid analogs as in the case of Light-up probes.

Light-up probes are peptide nucleic acids (PNAs) oligonucleotide. They are linked with thiazole orange as the fluorophore and no quencher is required (Svanvik et al., 2000). PNA molecules have a backbone with peptide like covalent bonds and exocyclic bases. When light-up probes hybridize with specific target DNA, the resulting duplex or triplex structures elicit increased fluorescence of the fluorophore (Isacsson et al., 2000).

6. Real time quantification

The quantification strategy is an important factor for detecting of mRNA gene expression level. Quantification of mRNA transcription can be measured by absolute or relative quantitative Real-time PCR (Souazé et al., 1996; Pfaffl, 2001a; Bustin, 2002). Absolute quantification is an analysis method to accurately measure the copy number of a target sequence (in picograms or nanograms of DNA or RNA) in the sample, while relative quantification provides relative changes in mRNA expression levels as a ratio of the amount of initial target sequence between control and analysed samples (Souazé et al., 1996; Pfaffl, 2001a; Fraga et al., 2008). Thus, relative quantification simply allows us to determine the fold changes between sample and control. If the purpose is accurately measuring the copy number of a target sequence, absolute quantification strategy which requires standards of known copy number, should be performed. Moreover, these standards should be amplified in the same run (Peirson et al., 2003).

Both approaches are generally used but relative quantification requires less set up time and easier to perform than absolute quantification because a standard curve is not essential (Livak, 2001; Pfaffl 2004b; Fraga et al., 2008). Furthermore, it is commonly not necessary to know the absolute amount of mRNA in biological applications examining gene expression (Bustin, 2002; Huggett et al., 2005).

6.1 Absolute quantification

This approach is more precise but often more labor-intensive (Bustin & Nolan, 2004a). Absolute quantification requires a standard calibration curve using serially diluted standards of known concentrations for highly specific, sensitive and reproducible data (Reischl & Kochanowski, 1995; Heid et al., 1996; Bustin, 2000; Pfaffl & Hageleit, 2001; Pfaffl, 2001b; Fraga et al., 2008). Linear relationship between Ct and initial amounts of total RNA or cDNA using standard curve allows the detection of unknowns' concentration based on their Ct values (Heid et al., 1996).

In this method, all standards and samples have equal amplification efficiency. It is necessary to control the efficiency of the Real-time PCR reaction to quantify mRNA levels (Fraga et al., 2008). Real-time PCR amplification efficiencies for calibration curve and target cDNA must have identical reverse transcription efficiency to provide a valid standard for mRNA quantification (Pfaffl & Hageleit, 2001). The amplification efficiencies of the standard and unknown target sequence should be approximately equal and the concentration of the serial dilutions should be within the range of the unknown(s) in order to ensure correct results.

The standard and target sequence should have the same primer binding sites and produce a product of approximately the same size and sequence (Fraga et al., 2008). The standard can be based on known concentrations of double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), commercially synthesized big oligonucleotide and complementary RNA (cRNA) bearing the target sequence (Reischl & Kochanowski, 1995; Morrison et al., 1998; Bustin 2000; Kainz, 2000; Pfaffl & Hageleit, 2001; Pfaffl et al., 2001c; Wong & Medrano, 2005). DNA standards can be synthesized by cloning the target sequence into a plasmid (Gerard et al., 1998), purifying a conventional PCR product, or directly synthesizing the target nucleic acid (Liss, 2002). These standards have a property of larger quantification range, greater sensitivity, more reproducibility and higher stability than RNA standards (Pfaffl, 2004b).

However, DNA standards are generally not possible to use as a standard for absolute quantitation of RNA because there is no control for the efficiency of the reverse transcription step. (Livak, 2001; Wong & Medrano, 2005). Therefore, RNA molecules are strongly recommended as standards for quantification of RNA (Real Time PCR, 2010).

In standard preparation for RNA quantitation, an *in vitro*-transcribed sense RNA transcript is generated, the sample is digested with RNase-free DNase so that the RNA is quantitated accurately. Because any significant DNA contamination will result in inaccurate quantification (Bustin, 2000, 2002). A recombinant RNA (recRNA) can be synthesized *in vitro* by cloning the DNA of the gene of interest (GOI) into a suitable vector, containing typically SP6, T3, or T7 phage RNA polymerase promoters (Gibson et al., 1996; Pfaffl & Hageleit, 2001; Pfaffl, 2001b; Fronhoffs et al., 2002; Fraga et al., 2008). Several commercial kits are available that facilitate the production of RNA from these vectors. After *in vitro* transcribed RNA (standard RNA) is synthesized, the standard concentration is measured on a spectrophotometer and converted the absorbance to a 'target copy number per μg RNA' (Bustin, 2000). Once the standard has been accurately quantified, it is serially diluted in increments of 5- to 10-fold and each dilution should be run in triplicate (Fraga et al., 2008). The dilutions should be made over the range of copy numbers that include the likely amount of target mRNA expected to be present in the experimental samples to maximize accuracy (Bustin, 2000; Fraga et al., 2008). After dilutions generated, known amounts of RNAs are converted to cDNA for subsequent Real-time PCR. A standard curve is created by plotting the average Ct values (inversely proportional to the log of the initial copy number) from each dilution versus the absolute amount of standard present in the sample (Higuchi et al., 1993; Bustin, 2000; Fraga et al., 2008).

As shown in Fig. 6, the copy numbers of sample RNAs can be calculated via comparison of samples' Ct values to this standard curve after real time amplification (Bustin, 2000). Under certain circumstances, absolute quantification models can also be normalized using suitable and unregulated references or housekeeping genes (Pfaffl, 2004b). Standard design, production, determination of the exact standard concentration, and stability over long storage time are tortuous and can be difficult (Bustin & Nolan, 2004a). In addition, the primary limitation to this approach is the necessity of obtaining an independent reliable standard for each gene. Moreover, running concurrent standard curves during each assay is needed.

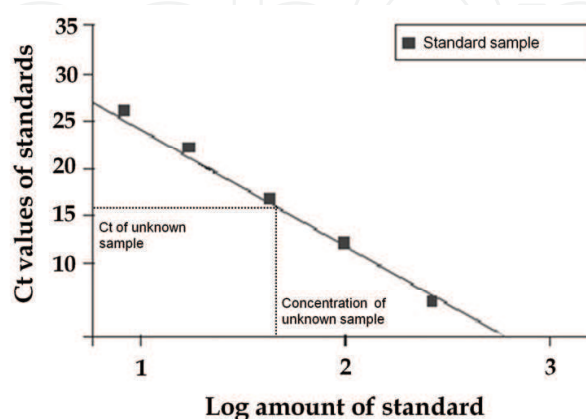


Fig. 6. Absolute quantification with standard curve. It shows determination of concentration of unknown sample.

6.2 Relative quantification

Relative quantification determines the changes in steady-state mRNA levels of GOI in response to different treatments (e.g., control versus experimental) or state of the tissue (e.g., infected versus uninfected samples, different developmental states or benign versus malign tissue) (Pfaffl et al., 2002a; Bustin & Nolan, 2004a; Valasek & Repa, 2005;). The advantage of using a relative quantification approach is that standards with known concentrations are not required so that there is no need for generating a standard calibration curve (Pfaffl 2004b; Fraga et al., 2008). In this approach, only relative changes can be determined because of the unknown internal standard quantity (Valasek & Repa, 2005;). This may not pose a problem for more research projects because absolute value of mRNA is commonly not necessary and fold change is adequate for investigating physiological changes in gene expression levels in many biological applications (Bustin, 2002; Pfaffl, 2004b; Huggett et al., 2005; Valasek & Repa, 2005). During relative quantification, amounts of target and reference gene's (sometimes called a housekeeping gene or internal control) are determined within the same sample. Housekeeping gene selection is an important issue and has been discussed in separate title (see housekeeping gene selection). After reaction, the Ct ratio between each target and the reference gene is calculated (Real-time PCR, 2010). The housekeeping gene which helps to normalize the data for experimental error, can be co-amplified in the same tube in a multiplex assay or can be amplified in a separate tube (Wittwer et al., 2001; Pfaffl et al., 2002a; Fraga et al., 2008).

6.3 Amplification efficiency

Amplification efficiency is an important factor for accurate relative quantification. In an optimal PCR reaction (100% efficient), every amplicon will be replicated and the amount of product will double after each cycle and a plot of copy number versus cycle number produces a line. However, if the reaction is only 90% efficient, the amount of product will not double after each cycle and the slope of the plot will be less than the same plot assuming 100% efficiency (Fraga et al., 2008). As mentioned earlier, the amplification efficiency is assumed an ideal or 1 (Gibson et al., 1996). However, small efficiency differences between target and reference gene result in inaccurate expression ratio (over or under initial mRNA amount instead of real). Difference in PCR efficiency (ΔE) of 3% ($\Delta E = 0.03$) between target gene and reference gene generates a falsely calculated expression ratio of 47% in case of $E_{\text{target}} < E_{\text{ref}}$ and 209% in case of $E_{\text{target}} > E_{\text{ref}}$ after 25 PCR cycles (Pfaffl et al., 2002b; Rasmussen, 2001). The amplification efficiencies of the target gene and housekeeping gene are preferred to be the same so that relative expression values for the target gene in samples are accurately compared (Schmittgen et al, 2000; Fraga et al., 2008). However, it is difficult to achieve identical amplification efficiencies in all PCRs. Therefore, lack of an appropriate correction factor might result in overestimation of the target gene's starting concentration (Liu & Saint, 2002a).

Traditionally, the amplification efficiencies of a genes (for example; target or reference genes) can be determined by preparing a 10-fold dilution series from a reference RNA or cDNA sample and by plotting the Ct as a function of $\log_{10}$ concentration of template. The slope of the resulting trend line (S) will be a clue of the PCR efficiency. Simply, amplification efficiency of a reaction is calculated using data collected from a standard curve plot with the following formula (Rasmussen, 2001):

$$\text{Exponential amplification} = 10^{(-1/S)} \quad (1)$$

$$E = [10^{(-1/S)}] - 1 \quad (2)$$

In above formulas, “E” refers to the efficiency of the reaction and “S” refers to the slope of the standard curve plot generated by Ct value versus the log of the input template amount.

A slope of -3.32 indicates the PCR reaction is 100% efficient. When a slope value is between -3.6 and -3.1, amplification efficiency ranges from 90% to 110% (e. g., $E = 0.9 - 1.1$). The appropriate efficiency of the PCR should be 90-110%. (Rasmussen, 2001; Tichopad et al., 2003). Theoretically, 3.3 cycles are required in order to increase the amplicon concentration 10-fold when a PCR reaction proceeding at 100% efficiency. Additionally, a Ct alteration of 1 between different samples corresponds to a 2-fold changes in starting material (Fraga et al., 2008).

Amplification efficiency depends on many factors, such as efficiency of primer annealing, the length of the amplicon, GC content of the amplicon and sample impurities (McDowell et al., 1998). These factors affect primer binding, the melting point of the target sequence, and the processivity of the DNA polymerase. (Wiesner, 1992). Therefore, the target gene and the reference gene’s amplification efficiencies are usually found different. Thus, determination of the amplification efficiencies of analysed genes should be done carefully in Real-time PCR assays.

6.4 Data analysis methods and software applications

The Ct values obtained from Real-time PCR analysis need to be converted using different procedure in order to make valid comparisons (Fraga et al., 2008). Besides, the classical Real-time PCR parameters (i.e. primer design, RNA quality, reverse transcription and polymerase performances), Real-time PCR data processing can influence or even change the final results. Analysis of Real-time PCR data can be either of absolute levels (i.e., numbers of copies of a specific RNA per sample) or relative levels.

In absolute quantification, the Ct value for each sample should be compared with standard curve to extrapolate the starting concentration. (Wilkening & Bader, 2004; Fraga et al., 2008; Vanguilder et al., 2008). Besides, the absolute gene quantification strategy, the relative expression strategy compares GOI in relation to a reference gene, is commonly used by the academic research community.

To date, several mathematical models using for calculating relative expression ratio (R) or fold induction have been developed and they are based on the comparison of the distinct cycle differences (Meijerink et al., 2001; Pfaffl, 2001a; Liu and Saint, 2002b). Two types of relative quantification models are available and used generally;

A) Relative quantification without efficiency correction or the Comparative Ct method;

The comparative Ct method is a mathematical model based on the delta-Ct (ΔCt) (Wittwer et al., 2001) or delta-delta-Ct ($\Delta\Delta Ct$) values in most applications, described by Livak and Schmittgen (Livak & Schmittgen, 2001) without efficiency correction. In this model, an optimal doubling of the target sequence during each performed Real-time PCR cycle is assumed (Winer et al., 1999; Livak, 2001; Livak & Schmittgen, 2001). This analysis can be

performed in two ways; Non-normalized expression (also known as ΔC_t method) and normalized expression (also known as $\Delta\Delta C_t$ method).

A1) Non-normalized Expression (ΔC_t) method; In relative quantification, a comparison is made with the gene expressed in the sample to that of the same gene expressed in the control. C_t values are non-normalized using housekeeping gene, but normalization is accomplished via equal loading of samples. Quantitation is performed relative to the control by subtracting the C_t value of the control gene from C_t of the sample gene (ΔC_t). The fold difference of target gene in sample and control is calculated by using the resulting differences in cycle number (ΔC_t) as the exponent of the base 2 (due to the doubling function of PCR) as given below in eq. 3 and 4.

$$R = 2^{\Delta C_t} \quad (3)$$

$$R = 2^{[C_t \text{ sample} - C_t \text{ control}]} \quad (4)$$

A2) Normalized Expression ($\Delta\Delta C_t$) method; In this approach, loading differences are eliminated. Moreover, the C_t values of both the control and the samples for target gene are normalized to an appropriate housekeeping or reference gene. This method also known as $2^{-\Delta\Delta C_t}$ method, where $\Delta\Delta C_t = \Delta C_t \text{ sample} - \Delta C_t \text{ control}$. Formulas are given below in eq.5 and 6.

$$R = 2^{-\Delta\Delta C_t} \quad (5)$$

$$R = 2^{-[\Delta C_t \text{ sample} - \Delta C_t \text{ control}]} \quad (6)$$

$$\Delta C_t (\text{sample}) = C_t \text{ target gene} - C_t \text{ reference gene}$$

$$\Delta C_t (\text{control}) = C_t \text{ target gene} - C_t \text{ reference gene}$$

$$\Delta\Delta C_t = \Delta C_t (\text{sample}) - \Delta C_t (\text{control})$$

The reaction is rigorously optimized and the PCR product size should be kept small (less than 150 bp) (Marino et al., 2003; Wong & Medrano, 2005). Comparative C_t method can be chosen when assaying a large number of samples because the standard curve is unnecessary (Wong & Medrano, 2005).

This model is acceptable for a first approximation of the crude expression ratio. However, efficiency (E) corrected models are useful to obtain reliable relative expression data (Pfaffl et al., 2009).

B) Relative quantification with efficiency correction or Pfaffl model: Pfaffl developed a mathematical formula widely used for the relative quantification of gene expression in Real-time PCR (Pfaffl, 2001a). This model combines gene quantification and normalization with an amplification efficiency of the target and reference genes. This calculation can be based on one sample (Souazé et al., 1996;; LightCycler® Relative Quantification Software, 2001) or multiple samples (Pfaffl, 2001a,, 2004b) and their formulas are given in Eqs. 7-8 and 9, respectively. Reactions for the determination of efficiencies of the genes should be run in a 5 or 10-fold serially diluted sample.

$$R = \frac{(E_{\text{target}})^{\Delta C_{\text{t target}} (\text{control} - \text{sample})}}{(E_{\text{ref}})^{\Delta C_{\text{t ref}} (\text{control} - \text{sample})}} \quad (7)$$

$$R = \frac{(E_{\text{ref}})^{C_{\text{t sample}}}}{(E_{\text{target}})^{C_{\text{t sample}}}} \div \frac{(E_{\text{ref}})^{C_{\text{t control}}}}{(E_{\text{target}})^{C_{\text{t control}}}} \quad (8)$$

$$R = \frac{(E_{\text{target}})^{\Delta C_{\text{t target}} (\text{MEAN}_{\text{control}} - \text{MEAN}_{\text{sample}})}}{(E_{\text{ref}})^{\Delta C_{\text{t ref}} (\text{MEAN}_{\text{control}} - \text{MEAN}_{\text{sample}})}} \quad (9)$$

In new approaches, multiple reference genes is used to obtain more stable and reliable results (Vandesompele et al., 2002). An efficiency corrected calculation models, based on multiple samples and reference genes (so-called REF index), should consist of at least three reference genes (eq. 10) (Pfaffl, 2004b).

$$R = \frac{(E_{\text{target}})^{\Delta C_{\text{t target}} (\text{MEAN}_{\text{control}} - \text{MEAN}_{\text{sample}})}}{(E_{\text{ref index}})^{\Delta C_{\text{t ref index}} (\text{MEAN}_{\text{control}} - \text{MEAN}_{\text{sample}})}} \quad (10)$$

Analysis of the raw data in precise mathematical and statistical manner should be performed rationally in gene expression analysis. Various software tools and excel spreadsheets are available to calculate the raw data. The LightCycler relative expression software (Roche Applied Science), Q-Gene (Muller et al., 2002), qBASE (Hellemans et al., 2007), SoFar (Wilhelm et al., 2003), DART (Peirson et al., 2003), qPCR-DAMS (Jin et al., 2006) and REST software applications (Pfaffl et al., 2002b) can be used for calculation. Only Q-Gene (Muller et al., 2002) and REST (Pfaffl et al., 2002b) software packages are freely available. Q-Gene uses a paired or an unpaired Student's *t* test, a Mann-Whitney U-test, or Wilcoxon signed-rank test (Muller et al., 2002).

The REST software established in 2002 performs Pair-Wise Fixed Reallocation Randomization Test which repeatedly and randomly reallocates at least 2000 times the observed Ct values to the two groups (Pfaffl et al., 2002b; Pfaffl et al., 2004b). Two new version of REST software package (REST 2008 and REST 2009) were developed by Pfaffl and co-workers and the single run efficiency is implemented in REST 2008 as well as multiple reference gene normalization. In REST 2009, randomization algorithms have been improved to obtain better confidence intervals and more accurate *p* values. Moreover, the best fit for the standard curve is used for the determination of the efficiency and it is used in the randomization process.

7. Housekeeping gene selection

The proper housekeeping gene (HKG) is continuously expressed in all cell types and tissues (Thellin et al., 1999). Additionally, the expression level of a suitable reference gene should be stable and is not affected by the biologic and experimental condition or by the disease state (Vandesompele et al., 2002). Nevertheless, there is no universal housekeeping gene having invariable expression under all these circumstances (Thellin et al., 1999). Therefore, choosing

a stable housekeeping gene is crucial for the accurate interpretation of gene expression data. (Zhang et al., 2005). Furthermore, using more than one HKG is recommended for the convenient results. The most frequently used housekeeping genes involved β -actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyl transferase 1 (HPRT1), β 2-microglobulin (B2M), phosphoglycerokinase1 (PGK1), cyclophilin A (CPA) and 18S rRNA.

GeNorm (Vandesompele et al., 2002) (available at <http://medgen.ugent.be/~jvdesomp/genorm/>), Normfinder (Andersen et al., 2004) (available at <http://www.mdl.dk/publicationsnormfinder.htm>) and Bestkeeper (Pfaffl et al. 2004a) (available at <http://www.gene-quantification.de/bestkeeper.html>) programs are used to determination of housekeeping gene mRNA expression stability.

There are numerous studies on the selection of the proper reference gene in many different tissues and cell types. Calcagno et al. suggested that plasma membrane calcium-ATPase 4 (PMCA4) is a suitable reference gene for normalization of gene expression for polytopic membrane proteins including transporters, ATPases and receptors (Calcagno et al., 2006). Cicinnati et al. showed that hydroxymethyl-bilane synthase (HMBS) and GAPDH are good reference genes for normalizing gene expression data between paired tumoral and adjacent non-tumoral tissues derived from patients with human hepatocellular carcinoma (HCC) (Cicinnati et al., 2008). It is shown that TATA box binding protein (TBP) and HPRT1 are the most reliable reference genes for q-PCR normalization in HBV related HCCs' matched tumor and non-tumor tissue samples (Gao et al., 2008; Fu et al., 2009). The cyclophilin A gene [peptidylprolyl isomerase A gene (PPIA)] is recommended as a housekeeping gene for gene expression studies in atopic human airway epithelial cells (AEC) of asthmatics (He et al., 2008). Penna et al. suggested that the use of two reference genes [Eukaryotic translation initiation factor 4A2 (EIF4A2) and Cytochrome c-1 (CYC1)] is proper for the normalization of the RT-qPCR data in human brain tissues (Penna et al., 2011). Pfister et al. demonstrated that the ribosomal protein L37A (RPL37A) is the most ideal housekeeping gene in meningiomas and their normal control tissue arachnoidea, dura mater and normal brain. The use of the combination of RPL37A and eukaryotic translation initiation factor 2B, subunit 1 alpha (EIF2B1) housekeeping genes is also recommended (Pfister et al., 2011). In another study, it is shown that the best choice of a reference gene for expression studies on astrocytomas is GAPDH. If two genes are used for gene normalization, authors recommend the combination of ribosomal protein, large, P0 (RPLP0) and histone cluster 1 (H3F). (Gresner et al., 2011).

Silver et al. showed that GAPDH is the most suitable HKG in reticulocyte studies (Silver et al., 2006). It is shown that succinate dehydrogenase complex subunit A (SDHA) is the best individual reference gene in neonatal human epidermal keratinocytes after UVB exposure. Also, SDHA and PGK1 were designated as the best combination (Balogh et al., 2008).

8. Normalization

Data normalization is a further major step for quantification of target gene expression in Real-time PCR (Pfaffl 2001a; Bustin, 2002). Appropriate normalization strategies are required to correct errors in Real-time PCR (Huggett et al., 2005; Wong & Medrano, 2005). These errors can be originated from a number of factors (variation in RNA integrity, sample-

to-sample variation, PCR efficiency differences, cDNA sample loading variation etc.) (Karge et al., 1998; Mannhalter et al., 2000). Performing a normalization strategy is also crucial to control for the amount of starting material, variation of amplification efficiencies and differences between samples. However, this remains the most intractable problem for real-time quantification (Thellin et al., 1999). Starting material usually varies in tissue mass, cell number or experimental treatment. mRNA levels can be standardized to cell number under ideal conditions in *in vitro* model. Ensuring similar tissue volume or weight appear to be straightforward, but this type of normalization is not possible because it can be difficult to ensure that different samples contain the same cellular material (Vandesompele et al., 2002).

Several strategies can be chosen for normalising Real-time PCR data including reference gene selection, similarity of sample size and quality of RNA (Huggett et al., 2005). Precise quantification and good quality of RNA is essential prior to reverse transcription (Bustin & Nolan, 2004b). Data normalization can be carried out against an endogenous unregulated reference gene transcript or against total cellular RNA content (molecules/g total RNA and concentrations/g total RNA) but normalization to total RNA is unreliable. Because knowledge about the total RNA content or even about the mRNA concentrations of the cells can not be accurately determined (Bustin, 2000, 2002). Normalising strategy using a housekeeping gene is a simple and convenient method for correction of sample-to-sample variation in Real-time PCR. Target and housekeeping gene expression levels should be within a similar range. For example, HPRT gene expression is very low in most human tissues so that this gene is only suitable for the normalization of lowly expressed target genes (Huggett et al., 2005).

Although it is best to start with the same amount of RNA concentration in cDNA synthesis step, sometimes this can not be achieved due to pipetting errors. Such an error can be partly controlled by using reference genes (Huggett et al., 2005).

9. Conclusion

In summary, qPCR is rapid, cost-effective, accurate, sensitive, reliable and reproducible method so that this technology has become a routine and robust approach for nucleic acid-based diagnostics and research area. It is frequently used for the analysis of gene expression profiles, the discovery of novel and surrogate molecular biomarkers of disease and validation of microarray data. Real-time PCR technique is preferred by numerous research labs around the world. While convenient normalisation and choosing an appropriate housekeeping gene are critical for obtaining biologically relevant results, an ideal normalisation remains to be answered in a satisfactory manner.

10. References

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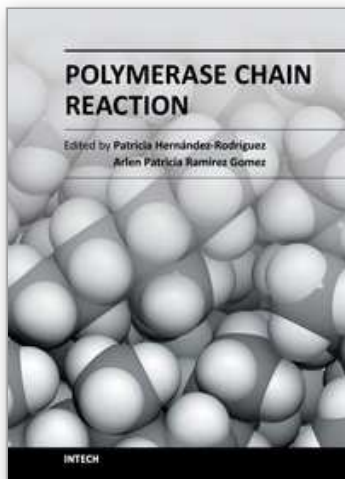
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