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Overview of Real-Time PCR Principles

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1. Introduction

Real-time PCR is based on the revolutionary method of PCR, developed by Kary Mullis in the 1980s, which allows researchers to amplify specific pieces of DNA more than a billion-fold (Saiki, Scharf et al. 1985; Mullis and Faloona 1987; Mullis 1990). PCR-based strategies have propelled molecular biology forward by enabling researchers to manipulate DNA more easily, thereby facilitating both common procedures, such as cloning, and huge endeavors such as the Human Genome Project (Olson, Hood et al. 1989; Ausubel, Brent et al. 2005). Real-time PCR represents yet another technological leap forward that has opened up new and powerful applications for researchers throughout the world. This is in part because the enormous sensitivity of PCR has been coupled to the precision afforded by "real-time" monitoring of PCR products as they are generated (Valasek and Repa 2005).

Higuchi and co-workers (Higuchi, Dollinger et al. 1992; Higuchi, Fockler et al. 1993) at Roche Molecular Systems and Chiron accomplished the first demonstration of real-time PCR. By including a common fluorescent dye called ethidium bromide (EtBr) in the PCR and running the reaction under ultraviolet light, which causes EtBr to fluoresce, they could visualize and record the accumulation of DNA with a video camera. It has been known since 1966 that EtBr increases its fluorescence upon binding of nucleic acids (Le Pecq and Paoletti 1966), but only by combining this fluorescent chemistry with PCR and real-time videography could real-time PCR be born as it was in the early 1990s. Subsequently, this

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technology quickly matured into a competitive market, becoming commercially widespread and scientifically influential (Valasek and Repa 2005).

Real-time PCR instrumentation was first made commercially available by Applied Biosystems in 1996, after which several other companies added new machines to the market. Presently, Applied Biosystems, BioGene, Bioneer, Bio-Rad, Cepheid, Corbett Research, Idaho Technology, MJ Research, Roche Applied Science, and Stratagene all offer instrumentation lines for real-time PCR (BioInformatics 2003).

Widespread use has also resulted in a multiplicity of names for the technology, each with a different shade of meaning. Real-time PCR simply refers to amplification of DNA (by PCR) that ismonitored while the amplification is occurring. The benefit of this real-time capability is that it allows the researcher to better determine the amount of starting DNA in the sample before the amplification by PCR. Present day real-time methods generally involve fluorogenic probes that "light up" to show the amount of DNA present at each cycle of PCR. "Kinetic PCR" refers to this process as well. "Quantitative PCR" refers to the ability to quantify the starting amount of a specific sequence of DNA. This term predates real-time PCR because it can refer to any PCR procedure, including earlier gel-based end-point assays, that attempts to quantify the starting amount of nucleic acid. Rarely, one might see the term "quantitative fluorescent PCR" to designate that the quantification was accomplished via measuring output from a fluorogenic probe, although this is redundant because all of the present chemistries for real-time PCR are fluorescent. In addition, if reverse transcriptase enzymes are used before PCR amplification in any of the above situations, then "RT-PCR" replaces "PCR" in the term. Today, the two most common terms, real-time and quantitative, are often used interchangeably or in combination, because real-time PCR is quickly becoming the method of choice to quantify nucleic acids (Valasek and Repa 2005).

The basic goal of real-time PCR is to precisely distinguish and measure specific nucleic acid sequences in a sample even if there is only a very small quantity. Real-time PCR amplifies a specific target sequence ina sample then monitors theamplification progress using fluorescent technology. During amplification, how quickly the fluorescent signal reaches a threshold level correlates with the amount of original target sequence, thereby enabling quantification. In addition, the final product can be further characterized by subjecting it to increasing temperatures to determine when the double-stranded product "melts." This melting point is a unique property dependent on product length and nucleotide composition. To accomplish these tasks, conventional PCR has been coupled to state-of-the-art fluorescent chemistries and instrumentation to becomereal-time PCR (Valasek and Repa 2005).

2. The chemistries of real-time PCR

Today fluorescence is exclusively used as the detection method in real-time PCR. Both sequence specific probes and non-specific labels are available as reporters. In his initial work Higuchi used the common nucleic acid stain ethidium bromide, which becomes fluorescent upon intercalating into DNA (Higuchi, Dollinger et al. 1992). Classical intercalators, however, interfere with the polymerase reaction, and asymmetric cyanine dyes such as SYBR Green I and BEBO have become more popular (Bengtsson, Karlsson et al. 2003; Zipper, Brunner et al. 2004). Asymmetric cyanines have two aromatic systems containing nitrogen, one of which is positively charged, connected by amethine bridge. These dyes have virtually no fluorescence when they are free in solution due to vibrations engaging

both aromatic systems, which convert electronic excitation energy into heat that dissipates to the surrounding solvent. On the other hand the dyes become brightly fluorescent when they bind to DNA, presumably to the minor groove, and rotation around the methine bond is restricted (Nygren, Svanvik et al. 1998). In PCR the fluorescence of these dyes increases with the amount of double stranded product formed, though not strictly in proportion because the dye fluorescence depends on the dye: base binding ratio, which decreases during the course of the reaction. The dye fluorescence depends also to some degree on the DNA sequence. But a certain amount of a particular double-stranded DNA target, in the absence of significant amounts of other double-stranded DNAs, gives rise to the same fluorescence every time. Hence, the dyes are excellent for quantitative real-time PCR when samples are compared at the same level of fluorescence in absence of interfering DNA. Although minor groove binding dyes show preference for runs of AT base-pairs (Jansen, Norde´n et al. 1993), asymmetric cyanines are considered sequence non-specific reporters in real-time PCR. They give rise to fluorescence signal in the presence of any double stranded DNA including undesired primer–dimer products. Primer–dimer formation interferes with the formation of specific products because of competition of the two reactions for reagents and may lead to erroneous readouts. It is therefore good practice to control for primer–dimer formation. This can be done by melting curve analysis after completing the PCR. The temperature is then gradually increased and the fluorescence is measured as function of temperature. The fluorescence decreases gradually with increasing temperature because of increased thermal motion which allows for more internal rotation in the bound dye (Nygren, Svanvik et al. 1998). However, when the temperature is reached at which the double stranded DNA strand separates the dye comes off and the fluorescence drops abruptly (Ririe, Rasmussen et al. 1997). This temperature, referred to as the melting temperature, Tm, is easiest determined as the maximum of the negative first derivative of the melting curve. Since primer–dimer products typically are shorter than the targeted product, they melt at a lower temperature and their presence is easily recognized by melting curve analysis (Kubista, Andrade et al. 2006).

Labeled primers and probes are based on nucleic acids or some of their synthetic analogues such as the peptide nucleic acids (PNA) (Egholm, Buchardt et al. 1992) and the locked nucleic acids (LNA) (Costa, Ernault et al. 2004). The dye labels are of two kinds: (i) fluorophores with intrinsically strong fluorescence, such as fluorescein and rhodamine derivatives (Sjöback, Nygren et al. 1995), which through structural design are brought into contact with a quencher molecule, and (ii) fluorophores that change their fluorescence properties upon binding nucleic acids. Examples of probes with two dyes are the hydrolysis probes, popularly called Taqman probes (Holland, Abramson et al. 1991), which can be based either on regular oligonucleotides or on LNA (Braasch and Corey 2001), Molecular Beacons (Tyagi and Kramer 1996; Tyagi, Bratu et al. 1998), Hybridization probes (Caplin, Rasmussen et al. 1999), and the Lion probes (http://www.biotools.net). The dyes form a donor–acceptor pair, where the donor dye is excited and transfers its energy to the acceptor molecule if it is in proximity. Originally the acceptor molecule was also a dye, but today quencher molecules are more popular (Wilson and Johansson 2003). Energy transfer and quenching are distance dependent and structural rearrangement of the probe, or, in the case of hydrolysis probes, degradation, change the distance between the donor and acceptor and, hence, the fluorescence of the system (Kubista, Andrade et al. 2006).

Probes based on a single dye, whose fluorescence changes upon binding target DNA include the LightUp probes (Svanvik, Westman et al. 2000), AllGlo probes

(http://www.allelogic.com), Displacement probes (Li, Qingge et al. 2002), and the Simple probes (http://www.idahotech.com/itbiochem/simpleprobes.html).

Chemical modifications and alterations of the oligonucleotide backbone are employed in some probes to improve the binding properties to the target template. This makes it possible to use shorter probes, which is advantageous for the detection of targets with short conserved regions such as retroviruses. LightUp probes have a neutral peptide nucleic acid (PNA) backbone that binds to DNA with greater affinity than normal oligonucleotides (Kubista, Andrade et al. 2006).

The LightUp probes are 10–12 bases, which is short compared to normal oligonucleotide probes that are usually at least 25 bases (http://www.lightup.se). LNA-probes make use of modified nucleotides to enhance binding affinity. MGBprobes are hydrolysis probes with a minor groove binding molecule attached to the end of the probe to increase affinity for DNA, which makes it possible to use shorter probes (Kutyavin, Afonina et al. 2000). Examples of modified primers include: Scorpion primers (Whitcombe, Theaker et al. 1999), LUX primers (Nazarenko, Lowe et al. 2002), Ampliflour primers (Uehara, Nardone et al. 1999), and the QZyme system (BD QZymeTM Assays for Quantitative PCR, 2003). As long as a single target is detected per sample there is not much of a difference in using a dye or a probe. Assay specificity is in both cases determined by the primers. Probes do not detect primer–dimer products, but using non-optimized probe assays is hiding the problem under the rug. If primer–dimers form they cause problems whether they are seen or not. In probe based assays, particularly when high CT values are obtained, one should verify the absence of competing primer–dimer products (Kubista, Andrade et al. 2006). The traditional way is by gel electrophoresis. Recently, an alternative approach was proposed based on the BOXTO dye. BOXTO is a sequence non-specific doublestranded DNA binding dye that has distinct spectral characteristics to fluorescein and can be used in combination with FAM based probes. The BOXTO and the probe signals are detected in different channels of the real-time PCR instrument. While the probe reflects formation of the targeted product as usual, the BOXTO dye also reports the presence of any competing primer–dimer products, which can be identified by melting curve analysis (Lind, Stahlberg et al. 2006). The great advantage of probes is for multiplexing, where several products are amplified in the same tube and detected in parallel (Wittwer, Herrmann et al. 2001). Today multiplexing is mainly used to relate expression of reporter genes to that of an exogenous control gene in diagnostic applications (Mackya 2004), and for single nucleotide polymorphism (SNP) and mutation detection studies (Mhlanga and Malmberg 2001; Mattarucchi, Marsoni et al. 2005). Multiplex assays are more difficult to design because when products accumulate the parallel PCR reactions compete for reagents. To minimize competition limiting amounts of primers must be used. Also, primer design is harder, because complementarity must be avoided between all the primers. Multiplex assays can be based either on probes or on labeled primers, where labeled primers usually give rise to signal from primer–dimer products, while probes do not. The different probing technologies have their advantages and limitations. Dyes are cheaper than probes but they do not distinguish between products. Hairpin forming probes have the highest specificity, because the formation of the hairpin competes with the binding to mismatched targets. This makes them most suitable for SNP and multi-site variation (MSV) analysis (Bonnet, Tyagi et al. 1999). Hydrolysis probes require two-step PCR to function properly, which is not optimal for the polymerase reaction, and short amplicons are

necessary to obtain reasonable amplification efficiencies. But they are easier to design than hairpin forming probes and an 80% success rate was recently reported (Kubista 2004).

In summary, a 'good' probe, independent of chemistry, should have low background fluorescence, high fluorescence upon target formation (high signal to noise ratio), and high target specificity. The dyes' excitation and emission spectra are important parameters to consider when designing multiplex reactions. Spectral overlap in excitation and emission should be minimized to keep cross-talk to a minimum (Kubista, Andrade et al. 2006).

2.1 SYBR green I

SYBR green I binds to the minor groove of dsDNA, emitting 1,000-fold greater fluorescence than when it is free in solution (Wittwer, Herrmann et al. 1997). Therefore, the greater the amount of dsDNA present in the reaction tube, the greater the amount of DNA binding and fluorescent signal from SYBR green I. Thus any amplification of DNA in the reaction tube is measured (Valasek and Repa 2005).

2.2 BEBO

The minor groove binding asymmetric cyanine dye BEBO is tested as sequence nonspecific label in real-time PCR. The Fluorescence intensity of BEBO increases upon binding to double-stranded DNA allowing emission to be measured at the end of the elongation phase in the PCR cycle. BEBO concentrations between 0.1 and 0.4 mM generated sufficient Fluorescence signal without inhibiting the PCR. A comparison with the commonly used reporter dye SYBR Green I shows that the two dyes behave similarly in all important aspects. The dye has absorbance and emission wavelengths that can be detected on the FAM channel on most common real-time PCR platforms, and shows a strong fluorescence increase when bound to dsDNA. BEBO can be used as an unspecific dye for real-time PCR applications or other applications where staining of dsDNA is wanted (Bengtsson, Karlsson et al. 2003).

2.3 BOXTO

The unsymmetrical cyanine dyes BOXTO and its positive divalent derivative BOXTO-PRO were studied as real-time PCR reporting fluorescent dyes and compared to SYBR GREEN I (SG). Unmodified BOXTO showed no inhibitory effects on real-time PCR, while BOXTO-PRO showed complete inhibition, sufficient fluorescent signal was acquired when 0.5–1.0 µM BOXTO was used with RotorGene and iCycler platforms. Statistical analysis showed that there is no significant difference between the efficiency and dynamic range of BOXTO and SG (Ahmad 2007).

2.4 5' nuclease (TaqMan) probes

Hydrolysis probes (also called 5'-nuclease probes because the 5'-exonuclease activity of DNA polymerase cleaves the probe) offer an alternative approach to the problem of specificity. These are likely the most widely used fluorogenic probe format (Mackay 2004) and are exemplified by TaqMan probes. In terms of structure, hydrolysis probes are sequence- specific dually fluorophore-labeled DNA oligonucleotides (Valasek and Repa 2005). One fluorophore is termed the quencher and the other is the reporter. When the quencher and reporter are in close proximity, that is, they are both attached to the same

short oligonucleotide; the quencher absorbs the signal from the reporter (Valasek and Repa 2005). This is an example of fluorescence resonance energy transfer (also called Forster transfer) in which energy is transferred from a "donor" (the reporter) to an "acceptor" (the quencher) fluorophore. During amplification, the oligonucleotide is broken apart by the action of DNA polymerase (5′-nuclease activity) and the reporter and quencher separate, allowing the reporter's energy and fluorescent signal to be liberated. Thus destruction or hydrolysis of the oligonucleotide results in an increase of reporter signal and corresponds with the specific amplification of DNA (Valasek and Repa 2005). Examples of common quencher fluorophores include TAMRA, DABCYL, and BHQ, whereas reporters are more numerous (e.g., FAM, VIC, NED, etc). Hydrolysis probes afford similar precision as SYBR green I (Wilhelm, Pingoud et al. 2003), but they give greater insurance regarding the specificity because only sequence-specific amplification is measured. In addition, hydrolysis probes allow for simple identification of point mutations within the amplicon using melting curve analysis (Valasek and Repa 2005).

2.5 Molecular beacons

Molecular beacons are similar to TaqMan probes but are not designed to be cleaved by the 5' nuclease activity of Taq polymerase. These probes have a fluorescent dye on the 5' end and a quencher dye on the 3' end of the oligonucleotide probe. A region at each end of the molecular beacon probe is designed to be complementary to itself, so at low temperatures, the ends anneal, creating a hairpin structure. This integral annealing property positions the two dyes in close proximity, quenching the fluorescence from the reporter dye (Espy, Uhl et al. 2006). The central region of the probe is designed to be complementary to a region of the PCR amplification product. At high temperatures, both the PCR amplification product and probe are single stranded. As the temperature of the PCR is lowered, the central region of the molecular beacon probe binds to the PCR product and forces the separation of the fluorescent reporter dye from the quenching dye. The effects of the quencher dye are obviated and a light signal from the reporter dye can be detected. If no PCR amplification product is available for binding, the probe reanneals to itself, forcing the reporter dye and quencher dye together, preventing fluorescent signal (Espy, Uhl et al. 2006). Typically, a single molecular beacon is used for detection of a PCR amplification product and multiple beacon probes with different reporter dyes are used for single nucleotide polymorphism detection. By selection of appropriate PCR temperatures and/or extension of the probe length, molecular beacons will bind to the target PCR product when an unknown nucleotide polymorphism is present but at a slight cost of reduced specificity. There is not a specific temperature thermocycling requirement for molecular beacons, so temperature optimization of the PCR is simplified (Espy, Uhl et al. 2006).

2.6 FRET hybridization probes

FRET hybridization probes, also referred to as LightCyclerprobes; represent a third type of probe detection format commonly used with real-time PCR testing platforms. FRET hybridization probes are two DNA probes designed to anneal next to each other in a head-to-tail configuration on the PCR product. The upstream probe has a fluorescent dye on the 3' end and the downstream probe has an acceptor dye on the 5' end. If both probes anneal to the target PCR product, fluorescence from the 3' dye is absorbed by the adjacent acceptor dye on the 5' end of the second probe. The second dye is excited and emits light at a third

wavelength and this third wavelength is detected. If the two dyes do not align together because there is no specific DNA for them to bind, then FRET does not occur between the two dyes because the distances between the dyes are too great. A design detail of FRET hybridization probes is the 3' end of the second (downstream) probe is phosphorylated to prevent it from being used as a primer by Taq during PCR amplification. The two probes encompass a region of 40 to 50 DNA base pairs, providing exquisite specificity (Espy, Uhl et al. 2006). FRET hybridization probe technology permits melting curve analysis of the amplification product. If the temperature is slowly raised, eventually the probes will no longer be able to anneal to the target PCR product and the FRET signal will be lost. The temperature at which half the FRET signal is lost is referred to as the melting temperature of the probe system (Espy, Uhl et al. 2006). The Tm depends on the guanine plus cytosine content and oligonucleotide length. In contrast to TaqMan probes, a single nucleotide polymorphism in the target DNA under a hybridization FRET probe will still generate a signal, but the melting curve will display a lower Tm. The lowered Tm can be characteristic for a specific polymorphism underneath the probes; however, a lowered Tm can also be the result of any sequence difference under the probes. The target PCR product is detected and the altered Tm informs the user there is a difference in the sequence being detected. Generally, more than three base pair differences under a FRET hybridization probe prevent hybridization at typical annealing temperatures and are not detected (Espy, Uhl et al. 2006). This trait of FRET hybridization probes is advantageous in cases where the genome of the organism is known to mutate at a high frequency, such as with viruses. When a single or limited number (<3) of known polymorphisms occur between two similar targets, FRET hybridization probes can also be used for discriminating strains of organisms (Espy, Uhl et al. 2006). Like molecular beacons, there is not a specific thermocycling temperature requirement for FRET hybridization probes. Molecular beacons and FRET hybridization probes, unlike TaqMan probes, are both recycled (conserved) in each round of PCR temperature cycle. Also, for Molecular beacons and FRET hybridization probes, unlike TaqMan probes, fluorescent signal does not accumulate as PCR product accumulates after each PCR cycle (Espy, Uhl et al. 2006).

2.7 Scorpions

Scorpions combine the detection probe with the upstream PCR primer (Whitcombe, Theaker et al. 1999) and consist of a fluorophore on the 5′ end, followed by a complementary stem-loop structure (also containing the specific probe sequence), quencher dye, DNA polymerase blocker (a nonamplifiable monomer that prevents DNA polymerase extension), and finally a PCR primer on the 3′ end. The probe sequence contained within the hairpin allows the scorpion to anneal to the template strand, which separates the quencher for the fluorophore and results in increased fluorescence. Because sequence-specific priming and probing is a unimolecular event, scorpions perform better than bimolecular methods under conditions of rapid cycling such as the LightCycler (Thelwell, Millington et al. 2000). Cycling is performed at a temperature optimal for DNA polymerase activity instead of the reduced temperature necessary for the 5′ nuclease assay. Scorpions are specific enough for allele discrimination and may be multiplexed easily (Thelwell, Millington et al. 2000). The scorpion chemistry has been improved with the creation of duplex scorpions in which the reporter dye/probe and quencher fragment are on separate, complementary molecules (Solinas, Brown et al. 2001). The duplex scorpions still bind in a unimolecular event, but because the reporter and quenchers are on separate molecules, they yield greater signal

intensity because the reporter and quencher can separate completely (Wong and Medrano 2005).

2.8 Sunrise™ primers

Created by Oncor (Gaithersburg, MD, USA), Sunrise primers are similar to scorpions in that they combine both the PCR primer and detection mechanism in the same molecule (Nazarenko, Bhatnagar et al. 1997). These probes consist of a dual-labeled (reporter and quencher fluorophores) hairpin loop on the 5′ end, with the 3′ end acting as the PCR primer. When unbound, the hairpin is intact, causing reporter quenching via FRET. Upon integration into the newly formed PCR product, the reporter and quencher are held far enough apart to allow reporter emission (Wong and Medrano 2005).

2.9 LUX™ fluorogenic primers

Light upon extension (LUX) primers (Invitrogen, Carlsbad, CA, USA) are self-quenched single-fluorophore labeled primers almost identical to Sunrise primers. However, rather than using a quencher fluorophore, the secondary structure of the 3′ end reduces initial fluorescence to a minimal amount (Nazarenko, Lowe et al. 2002). Because this chemistry does not require a quencher dye, it is much less expensive than dual-labeled probes. While this system relies on only two oligonucleotides for specificity, unlike the SYBR Green I platform in which a dissociation curve is used to detect erroneous amplification, no such convenient detection exists for the LUX platform. Agarose gels must be run to ensure the presence of a single PCR product, a step that is extremely important not only for the LUX primers but also for the Sunrise primers and scorpions because PCR priming and probe binding are not independent in these chemistries (Wong and Medrano 2005).

2.10 Light-up probes

Light-up probes are peptide nucleic acids (PNAs) that use thiazole orange as the fluorophor. Upon hybridisation with DNA, duplex or triplex structures are formed with increased fluorescence intensity of the fluorophor. A quencher is not required. This technique is limited by unspecific fluorescence, which increases during PCR and therefore restricts the achievable sensitivity (Isacsson, Cao et al. 2000; Svanvik, Stahlberg et al. 2000;Svanvik, Westman et al. 2000). Some other formats use the increasing quench as indicator for product accumulation (Crockett and Wittwer 2001; Kurata, Kanagawa et al. 2001). In this case, the fluorescence is quenched by a guanine residue of the PCR product. These probes are comparatively inexpensive and easy to construct; however, measurement of the decrease of a signal is problematic, especially during the early exponential phase in which only very few probes are quenched (Wilhelm and Pingoud 2003).

2.11 Eclipse probe

qPCR assays using an Eclipse probe employ two primers and a sequence-specific oligonucleotide probe. The probe is complementary to a sequence within the amplicon and contains a fluorescent reporter at the 3′ end, a quencher at the 5′ end, and a minor groove binder (Bio-Rad Laboratories 2006). The unhybridized probe adopts a conformation that brings the reporter and quencher together, quenching the reporter. During the annealing

step of PCR, the probe hybridizes to the target with the help of the minor groove binder. The probe thus becomes linearized, separating the reporter and quencher and allowing the reporter to fluoresce. The resulting fluorescent signal is proportional to the amount of amplified product in the sample (Bio-Rad Laboratories 2006).

2.12 Amplifluor primer

qPCR assays using Amplifluor chemistry employ two target-specific primers and one universal primer called the UniPrimer. The first target-specific primer contains a 5' extension sequence called the Z-sequence that is also found at the 3' end of the UniPrimer. The UniPrimer forms a hairpin structure (Bio-Rad Laboratories 2006). A fluorescent reporter and a quencher are attached at the 5' and the 3' ends of the stem structure, respectively. In the hairpin conformation, the reporter fluorescence is quenched due to its proximity to the quencher. During the first amplification cycle, the first target-specific primer (with the Z-sequence) hybridizes to the template and is extended. During the second amplification cycle, the second target-specific primer is used to synthesize a new target template that contains a sequence complementary to the Z-sequence. The product from the second amplification cycle can then serve as the template for the UniPrimer. In the third amplification cycle, the extended UniPrimer serves as a template for the next amplification cycle (Bio-Rad Laboratories 2006). In the fourth cycle, extension of the template through the hairpin region of the UniPrimer causes the UniPrimer to open up and adopt a linear configuration, which allows the reporter to fluoresce. Exponential amplification using the second target-specific primer and the UniPrimer occurs in subsequent amplification cycles. The resulting fluorescent signal is proportional to the amount of amplified product in the sample (Bio-Rad Laboratories 2006).

2.13 BD QZyme primer

qPCR assays using BD QZyme primers employ a target-specific zymogene primer, a target-specific reverse primer, and a universal oligonucleotide substrate. The oligonucleotide contains a fluorescent reporter on the 5' end and a quencher on the 3' end. When oligonucleotide substrate is intact, the fluorescence of the reporter is quenched by the quencher due to their proximity (Bio-Rad Laboratories 2006). The zymogene primer contains a sequence that encodes a catalytic DNA. During the first amplification cycle, the zymogene primer is extended. In the second cycle, the product of the first cycle is used as the template by the target-specific reverse primer, which is extended to create a new target sequence containing a catalytic DNA region. In the subsequent annealing step, the fluorescently labeled oligonucleotide substrate hybridizes to the catalytic DNA sequence and is cleaved. This cleavage separates the reporter from the quencher, resulting in a fluorescent signal that is proportional to the amount of amplified product in the sample (Bio-Rad Laboratories 2006).

3. Design and optimization of SYBR Green I reactions

A SYBR Green I assay uses a pair of PCR primers that amplifies a specific region within the target sequence of interest and includes SYBR Green 1 for detecting the amplified product. The steps for developing a SYBR Green I assay are:

1.   Primer design and amplicon design

2.   Assay validation and optimization

A) Primer and Amplicon Design:

A successful real-time PCR reaction requires efficient and specific amplification of the product. Both primers and target sequence can affect this efficiency. Therefore, care must be taken when choosing a target sequence and designing primers. A number of free and commercially available software programs are available for this purpose. One popular web-based program for primer design is Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). A commercially available program such as Beacon Designer software performs both primer design and amplicon selection (Bio-Rad Laboratories 2006).

B) Guidelines of amplicon design:

1.   Design amplicon to be 75–200 bp.Shorteramplicons are typically amplified with higher efficiency. An amplicon should be at least 75 bp to easily distinguish it from any primer-dimers that might form
2.   Avoid secondary structure if possible. Use programs such as mfold http://www.bioinfo.rpi.edu/applications/mfold/) to predict whether an amplicon will form any secondary structure at annealing temperature. See Real-Time PCR: General Considerations (Bio-Rad bulletin 2593) for more details
3.   Avoid templates with long (>4) repeats of single bases
4.   Maintain a GC content of 50–60%

C) Parameters of primer design:

1.   Design primers with a GC content of 50–60%
2.   Maintain a melting temperature (T_m) between 50ºC and 65ºC. We calculate T_m values using the nearest-neighbor method with values of 50 mM for salt concentration and 300 nM for oligonucleotide concentration
3.   Avoid secondary structure; adjust primer locations outside of the target sequence secondary structure if required
4.   Avoid repeats of Gs or Cs longer than three bases
5.   Place Gs and Cs on ends of primers
6.   Check sequence of forward and reverse primers to ensure no 3' complementarity (avoid primer-dimer formation)
7.   Verify specificity using tools such as the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/)

D) Assay Validation and Optimization:

Components a SYBR Green I qPCR reaction:

1.   PCR master mix with SYBR Green I
2.   Template
3.   Primers

Preformulated real-time PCR master mixes containing buffer, DNA polymerase, dNTPs, and SYBR Green I dye are available from several vendors.

Optimized SYBR Green I qPCR reactions should be sensitive and specific and should exhibit good amplification efficiency over a broad dynamic range (Bio-Rad Laboratories 2006).

Steps of to determine the performance of your SYBR Green I qPCR assay:

a.   Identify the optimal annealing temperature for your assay
b.   Construct a standard curve to evaluate assay performance

E) Annealing Temperature Optimization:

The optimal annealing temperature can easily be assessed on qPCR instruments that have a temperature gradient feature, such as the MiniOpticon™, MyiQ™, DNA Engine Opticon®, Opticon™ 2, iCycleriQ®, Chromo4™, and iQ™5 systems.

A gradient feature allows you to test a range of annealing temperatures simultaneously, so optimization reactions can be performed in a single experiment.

To find the optimal annealing temperature for reaction, recommend testing a range of annealing temperatures above and below the calculated T_m of the primers.

Because SYBR Green I binds to all dsDNA, it is necessary to check the specificity of your qPCR assay by analyzing the reaction product(s). To do this, use the melt-curve function on your real-time instrument and also run products on an agarose gel. An optimized SYBR Green I qPCR reaction should have a single peak in the melt curve, corresponding to the single band on the agarose gel.

Nonspecific products that may have been co-amplified with the specific product can be identified by melt-curve analysis. In this example, the specific product is the peak with a T_m of 89°C and corresponds to the upper band on the gel. The nonspecific product is the peak with a T_m of 78°C and corresponds to the lower band in the gel. By comparing the gel image with the melt curve, you can identify peaks in the melt curve that correspond to specific product, additional nonspecific bands, and primer-dimers. If nonspecific products such as primer-dimers are detected by melt-curve analysis, recommend that redesign primers (Bio-Rad Laboratories 2006).

F) Assay Performance Evaluation Using Standard Curves:

The efficiency, reproducibility, and dynamic range of a SYBR Green I assay can be determined by constructing a standard curve using serial dilutions of a known template. The efficiency of the assay should be 90–105%, the R^2 of the standard curve should be >0.980 or r > |–0.990|, and the C_T values of the replicates should be similar.

It is important to note that the range of template concentrations used for the standard curve must encompass the entire range of template concentration of the test samples to show that results from the test samples are within the linear dynamic range of the assay. If the test samples give results outside of the range of the standard curves, one of the following must be performed:

1.   Construct a wider standard curve covering the test sample concentrations and perform analysis to ensure that the assay is linear in that new range
2.   If the test samples give a lower C_T than the highest concentration of standards used in the standard curve, repeat the assay using diluted test samples
3.   If the test samples give a higher C_T than the lowest concentration of standards used in the standard curve, repeat the assay using larger amounts of the test samples

4. Design and optimization of TaqManProbe reactions

A TaqMan assay uses a pair of PCR primers and a dual-labeled target-specific fluorescent probe. The steps for developing a TaqMan assay are:

a.   Primer and probe design
b.   Assay validation and optimization

A. Primer and Probe Design:

As with any qPCR reaction, TaqMan-based assays require efficient and specific amplification of the product. Typically, the primers are designed to have an annealing temperature between 55 and 60°C. We recommend using software such as Beacon Designer for designing your TaqMan primers and TaqMan probe.. Because the dual-labeled probe is the most costly component of a TaqMan assay, suggested that order the two primers and validate their performance using SYBR Green I before ordering the dual-labeled probe.

The TaqMan probe should have a T_m 5–10°C higher than that of the primers. In most cases, the probe should be <30 nucleotides and must not contain a G at its 5' end because this could quench the fluorescent signal even after hydrolysis. Choose a sequence within the target that has a GC content of 30–80%, and design the probe to anneal to the strand that has more Gs than Cs (so the probe contains more Cs than Gs).

An important aspect of designing a TaqMan probe is reporter and quencher selection. We recommend using FAM-labeled probes when designing singleplex reactions, because they are inexpensive and readily available, perform well, and can be detected by all instruments currently on the market.

Another important consideration for obtaining accurate real-time qPCR data is probe quality. Even a perfectly designed probe can fail if the probe is improperly synthesized or purified. Improper removal of uncoupled fluorescent label, inefficient coupling, and/or poor quenching can produce high fluorescent background or noise. A low signal-to-noise ratio results in decreased sensitivity and a smaller linear dynamic range. Two probes with identical sequences and identical fluorophore labels can be measurably different when synthesized by different suppliers or even at different times by the same supplier.

B. Assay Validation and Optimization:

A TaqMan probe-based qPCR reaction contains the following components:

1.   PCR master mix
2.   Template
3.   Primers
4.   Probe(s)

Preformulated PCR master mixes containing buffer, DNA polymerase, and dNTPs are commercially available from several vendors. For TaqMan assays, we recommend using iQ™ supermix with 300 nM of each of the two primers and 200 nM of probe(s). TaqMan assays require careful attention to temperature conditions. A typical TaqMan protocol contains a denaturation step followed by a combined annealing and extension step at 55–60°C, instead of the traditional three-step PCR cycle of denaturation, annealing, and extension. This is to ensure that the probe remains bound to its target during primer

extension. Typical TaqMan probes for nucleic acid quantification are designed to have a T_m of 60–70°C. An optimized TaqMan assay should be sensitive and specific, and should exhibit good amplification efficiency over a broad dynamic range.

In short, construct a standard curve using dilutions of a known template and use this curve to determine the efficiency of the assay along with R^2 or r of the regression line. The efficiency of the reaction should be between 90 and 105%, the R^2 should be >0.980 or r > |– 0.990|, and the replicates should give similar C_T values. If the assay performs within these specifications, you are ready to start your experiment. If the assay performs outside these specifications, we suggest that you redesign your primers and TaqMan probe. It is important to note that the range of template concentrations used for the standard curve must encompass the entire range of template concentrations of the test samples to demonstrate that results from the test samples are within the dynamic range of the assay (Bio-Rad Laboratories 2006). If test samples give results outside the range of the standard curve, one of the three following steps must be performed:

1. Construct a wider standard curve covering the test sample concentrations and perform analysis to ensure that the assay is linear in that new range
2. If the test samples give a lower C_T than the highest concentration of standards used in the standard curve, repeat the assay using diluted test samples
3. If the test samples give a higher C_T than the lowest concentration of standards used in the standard curve, repeat the assay using larger amounts of the test samples

5. The instrumentation of real-time PCR

A critical requirement for real-time PCR technology is the ability to detect the fluorescent signal and record the progress of the PCR. Because fluorescent chemistries require both a specific input of energy for excitation and a detection of a particular emission wavelength, the instrumentation must be able to do both simultaneously and at the desired wavelengths. Thus the chemistries and instrumentation are intimately linked (Valasek and Repa 2005).

At present, there are three basic ways in which real-time instrumentation can supply the excitation energy for fluorophores: by lamp, light-emitting diode (LED), or laser. Lamps are classified as broad-spectrum emission devices, whereas LEDs and lasers are narrow spectrum. Instruments that utilize lamps (tungsten halogen or quartz tungsten halogen) may also include filters to restrict the emitted light to specific excitation wavelengths. Instruments using lamps include Applied Biosystem's ABI Prism 7000, Stratagene's Mx4000 and Mx3000P, and Bio-Rad's iCycleriQ. LED systems include Roche's LightCycler, Cepheid's SmartCycler, Corbett's Rotor-Gene, and MJ Research's DNA Engine Opticon 2. The ABI Prism 7900HT is the sole machine to use a laser for excitation (Valasek and Repa 2005). To collect data, the emission energies must also be detected at the appropriate wavelengths. Detectors include charge-coupled device cameras, photomultiplier tubes, or other types of photodetectors. Narrow wavelength filters or channels are generally employed to allow only the desired wavelength(s) to pass to the photodetector to be measured. Usually, multiple discrete wavelengths can be measured at once, which allows for multiplexing, i.e., running multiple assays in a single reaction tube (Valasek and Repa 2005). Another portion of the instrumentation consists of a thermocycler to carry out PCR. Of particular importance for real-time PCR is the ability of the thermocycler to maintain a

consistent temperature among all sample wells, as any differences in temperature could lead to different PCR amplification efficiencies. This is accomplished by using a heating block (Peltier based or resistive), heated air, or a combination of the two. As one might expect, heating blocks generally change temperature more slowly than heated air, resulting in longer thermocycling times. For example, Roche's LightCycler models utilizing heated air can perform 40 cycles in 30 min, whereas Applied Biosystem's ABI Prism 7900HT utilizing a Peltier-based heating block take s 1 h 45 min (Valasek and Repa 2005). Real-time instrumentation certainly would not be complete without appropriate computer hardware and data-acquisition and analysis software. Software platforms try to simplify analysis of real-time PCR data by offering graphical output of assay results including amplification and dissociation (melting point) curves. The amplification curve gives data regarding the kinetics of amplification of the target sequence, whereas the dissociation curve reveals the characteristics of the final amplified product (Valasek and Repa 2005).

5.1 Comparison of the different systems

Essentially, each real time PCR instrument consists of a computer-controlled thermocycler integrated with fluorescent detection system and dedicated software to analyze the result. Some systems can detect four different wave lengths (I-cycler, Mx4000 [stratagene] and Smart Cycler®, Version 2.0 Light Cycler®) whereas others can detect two different wavelengths (Light Cycler®). The Light Cycler® and Smart Cycler® are capable of performing rapid-cycle real time PCR because the reaction is set-up in capillaries or especially designated tubes. Both have optimized heating- cooling characteristic. A complete amplification protocol can be performed in 30-45 minutes (Myi ; Giulietti, Overbergh et al. 2001; Soheili and Samiei 2005). The Smart Cycler® is a combination of 16 individual, one tube real time PCR units. It is capable of performing a different PCR program on each of 16 reaction tubes. This is very useful for a rapid optimization of the assay as many variables can be tested at the same time. The Bio-Rad I-cycler IQ® instrument can perform real time amplification with a temperature gradient for specific PCR steps, allowing the optimization of real time PCR assay. The spectrofluorometers in the thermal cycler have a number of differences. Laser-based systems are tuned to excite each fluorophore at a specific wavelength and provide maximum efficiency. Lamp-based systems provide a broad excitation range that can be filtered to work with a number of fluorophores. The laser source not only gives brighter illumination to the fluorophore signal, but also produces less background noise(Myi ; Giulietti, Overbergh et al. 2001; Soheili and Samiei 2005).

In conclusion, real time PCR is a powerful advancement of the basic PCR technique. The important steps in deciding which particular assay format to use are related to the type of data required. The requirement for a research laboratory is quite distinct from those of a diagnostic laboratory. For the latter, probe confirmation of the PCR product is an essential part of the assay, whereas SYBR green detection may be sufficient for many other applications such as quantifying expression of a gene. All of the real-time PCR machines analyzed are capable of detecting PCR product in real time and a specific assay can be made optionally on every system. However, there are some decisions to be made when selecting among different formats. The choice of system is dependent on individual laboratory needs (Myi ; Giulietti, Overbergh et al. 2001; Soheili and Samiei 2005). Considering diagnostic applications, the Light Cycler® or Smart Cycler® may obtain faster results for urgent assays.

This could reduce the time of analysis to result from 3-4 hours to 1.5 hours. On the other hand, if sensitivity is the most important issue, these machines, with their smaller reaction volume and consequently lower sensitivity, wouldn't be the first choice. The ABI 7700 and Bio-Rad -I-Cycler IQ® have a 96 well format, enabling higher throughput than other systems. The 384-well plates, as designed by ABI for use in the 7900 HT system, can further enhance through put. For diagnostic application, internal control of nucleic acid isolation and PCR inhibition, it is essential to obtain valid results. This can be achieved using the system that enables multi-color detection, such as the I-Cycler IQ® and the Smart Cycler®. Recently, a multi-color format of the Light Cycler® is also present in market. Multiplex real-time PCRs can be developed for three different targets and an internal control by using the four detection wavelengths possible in multicolor detection. As a matter of fact, the choice of which real time system to use depends on the range of application required. To achieve meaningful results, each assay must be validated and optimized for the particular system chosen (Myi ; Giulietti, Overbergh et al. 2001; Soheili and Samiei 2005).

6. Advantages of real-time PCR quantitation

There are many methods in molecular biology for measuring quantities of target nucleic acid sequences. However, most of these methods exhibit one or more of the following shortcomings: they are time consuming, labor intensive, insufficiently sensitive, non-quantitative, require the use of radioactivity, or have a substantial probability of cross contamination (Reischl, Wittwer et al. 2002). These methods include but are not limited to Northern and Southern hybridizations, HPLC, scintillation proximity assay, PCR-ELISA, RNase protection assay, in situ hybridization, and various gel electrophoresis PCR end-point systems (Valasek and Repa 2005). Real-time PCR has distinct advantages over these earlier methods for several reasons. Perhaps the most important is its ability to quantify nucleic acids over an extraordinarily wide dynamic range (at least 5 log units). This is coupled to extreme sensitivity, allowing the detection of less than five copies (perhaps only one copy in some cases) of a target sequence, making it possible to analyze small samples like clinical biopsies or miniscule lysates from laser capture microdissection. With appropriate internal standards and calculations, mean variation coefficients are 1–2%, allowing reproducible analysis of subtle gene expression changes even at low levels of expression (Klein 2002; Luu-The, Paquet et al. 2005). In addition, all real-time platforms are relatively quick, with some affording high-throughput automation. Finally, real-time PCR is performed in a closed reaction vessel that requires no post-PCR manipulations, thereby minimizing the chances for cross contamination in the laboratory (Valasek and Repa 2005).

7. Limitations of real-time PCR quantitation

There are several limitations to real-time PCR methods. The majority of these are present in all PCR or RT-PCR-based techniques. Real-time PCR is susceptible to PCR inhibition bycompounds present in certain biological samples. For example, clinical and forensic uses for real-timePCR may be affected by inhibitors found in certain body fluidssuch as hemoglobin or urea (Wilson 1997). Food microbiological applications may encounter organic and phenolic inhibitors (Wilson 1997). To circumvent this problem, alternative DNA polymerases (e.g., Tfl, Pwo, Tth, etc.) that are resistant to particular inhibitors can be used. Other limitations primarily concern real-time PCR- based analysis of gene expression

(Bustin 2000; Bustin 2002;Bustin and Nolan 2004). Because of the necessary use of RNA in an extra enzymatic step, more problems have the opportunity to occur. RNA itself is extremely labile compared with DNA, and therefore isolation must be carefully performed to ensure both the integrity of the RNA itself and the removal of contaminating nucleases, genomic DNA, and RT or PCR inhibitors. This can be a problem with any sample source, but clinical samples are of special concern because inconsistencies in sample size, collection, storage, and transport can lead to a variable quality of RNA templates. Conversion of RNA to cDNA during the RT reaction is also subject to variability because multiple reverse transcriptase enzymes with different characteristics exist, and different classes of oligonucleotides (e.g., random, poly-dT, or gene specific primers) can be used to prime RT (Valasek and Repa 2005). Probably the largest present limitation of real-time PCR, however, is not inherent in the technology but rather resides in human error: improper assay development, incorrect data analysis, or unwarranted conclusions. In our experience using real-time PCR for gene expression analysis, real-time PCR primer sets must be designed and validated by stringent criteria to ensure specificity and accuracy of the results. For microbiology, false positives or negatives must be considered when designing an assay to detect pathogens. Amplification and melting curves must be visually inspected while independent calculations based on these curves should be double-checked for accuracy. Real-time PCR gene expression analysis measures mRNA levels and, therefore, only suggests possible changes in protein levels or function rather than demonstrating them. And although there is a tight connection between gene expression and gene product function (Brown and Botstein 1999)(8), this is certainly not always the case, and formal demonstration may be needed for a given research project. Of course, conclusions based on data derived from real-time PCR are best utilized when the biological context is well understood (Bustin 2002).

8. Types of real-time quantification

1. Absolute Quantitation

Absolute quantitation uses serially diluted standards of known concentrations to generate a standard curve. The standard curve produces a linear relationship between Ct and initial amounts of total RNA or cDNA, allowing the determination of the concentration of unknowns based on their Ct values (Heid, Stevens et al. 1996). This method assumes all standards and samples have approximately equal amplification efficiencies (Souaze, Ntodou-Thome et al. 1996). In addition, the concentration of serial dilutions should encompass the levels in the experimental samples and stay within the range of accurately quantifiable and detectable levels specific for both the real-time PCR machine and assay.The PCR standard is a fragment of double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), or cRNA bearing the target sequence (Wong and Medrano 2005). A simple protocol for constructing a cRNA standard for one-step PCR can be found in Fronhoffs et al. (Fronhoffs, Totzke et al. 2002), while a DNA standard for two-step real-time PCR can be synthesized by cloning the target sequence into a plasmid (Gerard, Olsson et al. 1998), purifying a conventional PCR product (Liss 2002), or directly synthesizing the target nucleic acid. The standard used must be a pure species. DNA standards have been shown to have a larger quantification range and greater sensitivity, reproducibility, and stability than RNA standards (Pfaffl, Tichopad et al. 2004). However, a DNA standard cannot be used for a one-step real-time RT-PCR due to the absence of a control for the reverse transcription efficiency (Giulietti, Overbergh et al. 2001).

2. Relative quantitation

During relative quantitation, changes in sample gene expression are measured based on either an external standard or a reference sample, also known as a calibrator (Livak and Schmittgen 2001). When using a calibrator, the results are expressed as a target/reference ratio. There are numerous mathematical models available to calculate the mean normalized gene expression from relative quantitationassays. Depending onthe method employed, these can yield different results and thus discrepant measures of standard error (Liu and Saint 2002; Muller, Janovjak et al. 2002). Table 1 shows a comparison of the different methods, with an explanation of each method to follow (Wong and Medrano 2005).

9. Quantitative analyses

For quantitative analysis, the amplification curves are evaluated. The amplification process is monitored either through the fluorescence of dsDNA-specific dyes (like SYBR Green I) or ofsequence-specific probes. Each curve consists of at least three distinct phases: 1) an initial lag phase in which no product accumulation can be measured, 2) an exponential phase, and 3) a plateau phase (Wilhelm and Pingoud 2003). The exponential phase in principle could be extrapolated to the start of the reaction (Cycle 0) to calculate the template copy number, but the error would be too high. The template copy number can be estimated with greater precision from the number of cycles needed for the signal to reach an arbitrary threshold. The threshold must intersect the signal curve in its exponential phase, in which the signal increase correlates with product accumulation. The intersection point is the so-called threshold value (C_T) or crossing point (C_P). This point may be between two successive cycles (i.e. it may be a fractional number). For exact quantifications, the efficiency of the amplification reaction must be known. It is crucial that the amplification efficiencies of standards and unknowns are identical (Wilhelm and Pingoud 2003). The efficiency can be estimated from the C_T values of samples with known template concentrations ('standards') as described below (Wilhelm and Pingoud 2003).

During the exponential phase, the signal *S* can be described by Equation 1:

$$S = pN_0\varepsilon^c \qquad (1)$$

where *p* is a proportionality factor to relate PCR product concentration and signal intensity, N_0 is the amount of template, *ε* is the amplification efficiency ($1 \leq \varepsilon \leq 2$; $\varepsilon = 2$ means 100% efficiency) and *c* is the cycle number.

Solving for c results in Equation 2:

$$c = -(\log\varepsilon)^{-1}(\log N_0 + \log p - \log S) \qquad (2)$$

With $m = -(\log\varepsilon)^{-1}$ and $b = -(\log\varepsilon)^{-1}(\log p - \log S)$, Equation 2 simplifies to Equation 3:

$$c = m\log N_0 + b \qquad (3)$$

This equation describes the linear relationship between the C_T values determined and the log of the template concentration (N_0). The parameters *m* and *b* can be determined by a regression analysis of the C_T values of the standards. When solved for N_0, this equation serves as a calibration curve for the calculation of the unknowns according to Equation 4:

$$N_0 = 10^{(C_T - b)/m} \qquad (4)$$

Methods (Reference)	Amplification Efficiency Correction	Amplification Efficiency Calculation	Amplification Efficiency Assumptions	Automated Excel-Based Program
Standard Curve (31)	no	standard curve	no experimental sample variation	no
Comparative C_t ($2^{-\Delta\Delta Ct}$) (21)	yes	standard curve	reference = target	no
Pfaffl et al. (26)	yes	standard curve	sample = control	REST[a]
Q-Gene (23)	yes	standard curve	sample = control	Q-Gene[b]
Gentle et al. (7)	yes	raw data	researcher defines log-linear phase	no
Liu and Saint (22)	yes	raw data	reference and target genes can have different efficiencies	no
DART-PCR (30)	yes	raw data	statistically defined log-linear phase	DART-PCR[c]

C_t, cycle threshold, DART-PCR, data analysis for real-time PCR; REST, relative expression software tool.

[a]www.gene-quantification.info
[b]www.BioTechniques.com
[c]nar.oupjournals.org/cgi/content/full/31/14/e73/DC1

Table 1. Characteristics of Relative Quantitation Methods

Detection Chemistries	Specificity	Multiplex Capability	Specific Oligonucleotide Required	Allelic Discrimination	Cost
DNA Binding Dyes	two PCR primers	No	No	No	$
Hybridization Probe Four Oligonucleotide Method	two PCR primers; two specific probes	Yes	Yes	Yes	$$$
Hybridization Probe Three Oligonucleotide Method	two PCR primers; one specific probe	Yes	Yes	Yes	$$$
Hydrolysis Probes	two PCR primers; one specific probe	Yes	Yes	Yes	$$$
Molecular Beacons	two PCR primers; one specific probe	Yes	Yes	Yes	$$$
Scorpions	one PCR primer; one primer/probe	Yes	Yes	Yes	$$$
Sunrise Primers	two PCR primers	Yes	Yes	Yes	$$$
LUX Primers	two PCR primers	Yes	Yes	No	$$

$$$, very expensive; $$, moderately expensive; $, inexpensive. LUX, light upon extension.

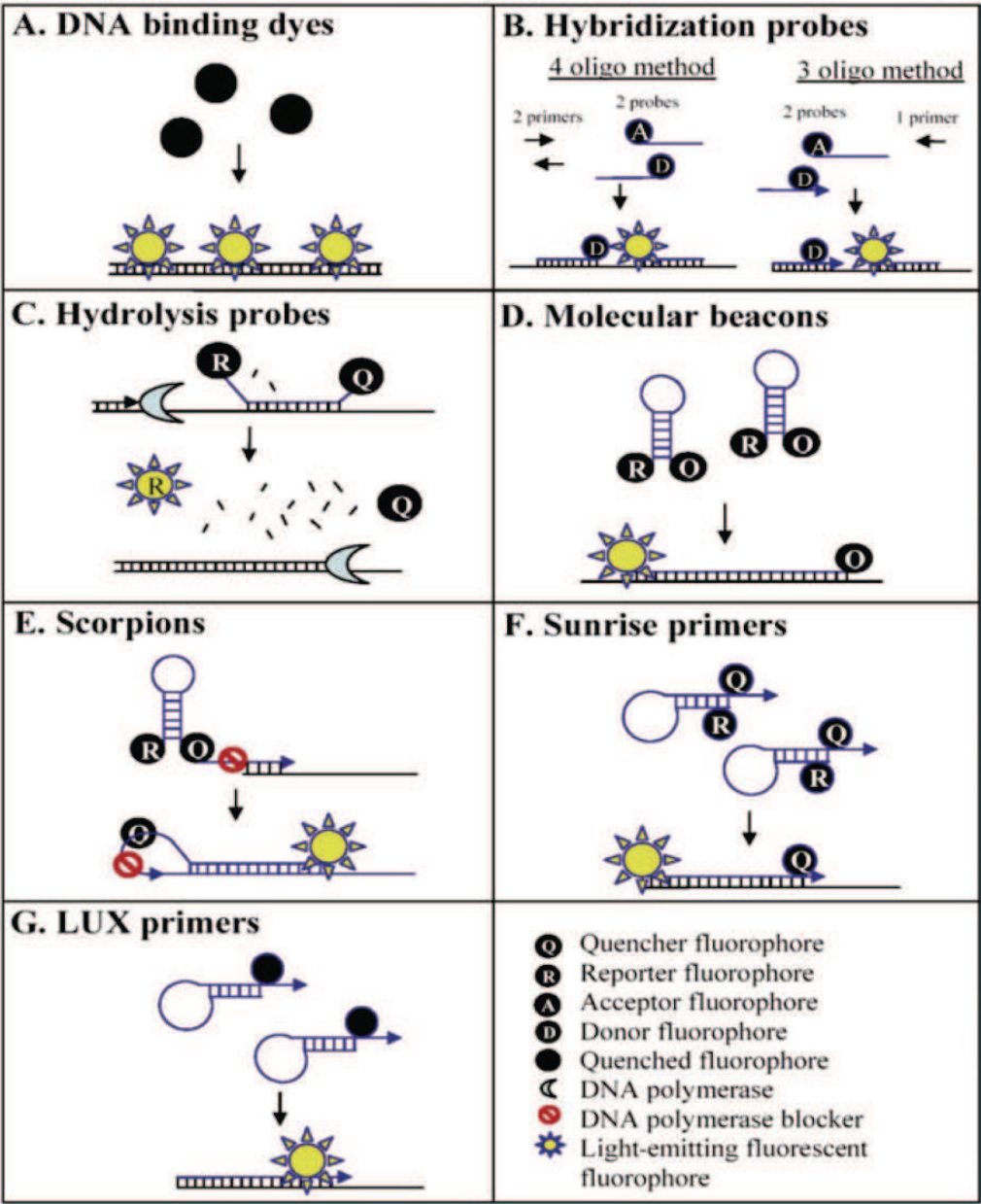Table 2. Characteristics of Detection Chemistries

Fig. 1. Real-time PCR detection chemistries. Probe sequences are shown in blue while target DNA sequences are shown in black. Primers are indicated by horizontal arrowheads. Not all unlabeled PCR primers are shown. Oligo, oligonucleotide.

The efficiency can be calculated from the parameter m by using Equation 5:

$$\varepsilon = 10^{-1/m} \tag{5}$$

By inserting ε back into Equation 4, one obtains Equation 6:

$$N_0 = \varepsilon^{(b-C_T)} \tag{6}$$

The maximum value for ε is 2.0 (i.e. the amount of product is doubled in each cycle). The experimental value for ε usually varies between 1.5 and 1.9. Lower efficiencies limit the sensitivity of the assay but allow quantifications with higher precisions. Therefore, reactions

should be optimized for high efficiency. The effect of the efficiency on the precision, however, is not pronounced.

With more than six orders of magnitude, the dynamic range of this procedure is extraordinarily high (Marcucci, Livak et al. 1998; Verhagen, Willemse et al. 2000;Sails, Fox et al. 2003). The accuracy of this technique is limited by the precision of the determination of the C_T values. The error of the C_T values results from the signal noise and the C_T calculation method. In highly optimized assays, standard errors of less than ± 0.2 cycles can be achieved. By assuming an amplification efficiency of 2 (i.e. 100 %), this implies that the minimum relative error for the quantification is about 10- 20%. The effects of different analysis and calculation methods and the effects of amplification-independent signal trends on the accuracy and precision of quantifications by realtime PCR are described in detail in papers by Lui et al. and Wilhelm et al (Liu and Saint 2002; Wilhelm, Pingoud et al. 2003).

Quantification is relative to the standard used. Only when the absolute concentration of the template molecules in the standard sample is known can the results be absolute. However, in most cases, determination of absolute concentrations is not required. That real-time PCR allows absolute quantification is demonstrated in principle by the reported determination of genome sizes (Wilhelm, Pingoud et al. 2003).

All quantifications by PCR are relative, either to a standard or to a reference gene. Interestingly, Equation 6 nicely illustrates the relative character of the quantifications using a dilution series of a standard; the meaning of the parameter b is the expected C_T value of a sample with 'one' copy (or any other unit as defined by the operator). The difference of this value minus the C_T value determined for the unknown sample ($\Delta C_T = b$ - C_T) is a direct measure for the relative difference in template concentrations of the unknown and standard (Wilhelm and Pingoud 2003).

To analyse relative changes in transcript levels, the chosen standard is usually a reference transcript, for example from a housekeeping gene, itself with unknown template concentration. The calculation of ΔC_T values between reference and sample transcript in a reference and a test sample then provides a simple tool to estimate relative changes. The derivation, assumptions and applications of the so-called $2^{\Delta\Delta CT}$ method are described elsewhere by Livak et al (Livak and Schmittgen 2001). The results of this method are only semiquantitative because the efficiency ε is assumed to be 2.0 in all experiments and for all templates, which is at best an optimistic estimate. More precise results are obtained with a procedure introduced by Pfaffl et al., which includes a measured value for ε (Pfaffl 2001; Wilhelm and Pingoud 2003).

In general, care must also be taken for accurate quantifications with external standardization, especially with respect to polymerase inhibitors, which may be present in differentconcentrations in the unknowns and standards. This problem is circumvented by internal standardization. Here, an analytically distinguishable standard template ('competitor') is added to the sample and co-amplified in the same reaction (Gilliland, Perrin et al. 1990; Goerke, Bayer et al. 2001). The direct and simultaneous quantitative analysis of both products in realtime PCR also poses problems. These difficulties are mostly due to the fact that different fluorophors have to be used to distinguish the sequences of competitor and sample. As a result of different FRET and quantum efficiencies, the C_T values obtained for competitor and sample are not directly comparable.

The problem of where to set the threshold makes relativequantifications difficult if not impossible. However, a simple trickcan be used to combine the advantages of both methods: thereaction mixtures are prepared in duplicate (Gibson, Heid et al. 1996). To one of thesemixtures, the probe specific for the competitor sequence isadded, whereas the probe specific for the sample sequence isadded to the other mixture. This process is carried out for a seriesof reactions with different amounts of competitor added. Withthis procedure, two calibration lines are obtained and theintersection of the two lines is the equivalence point (Wilhelm and Pingoud 2003).

10. Melting curve analyses

Melting curves represent the temperature dependence of the fluorescence. They are recorded subsequent to the amplification of the target sequence by PCR. The detection can be performed either with dsDNA-specific dyes like SYBR Green I or with sequence-specific probes such as the molecular beacons and the hybridisation probes (scorpion and sunrise primers cannot be used for melting curve analysis because they are integrated into the PCR products; TaqMan probes cannot be used for melting curve analyses either, since their signal generation depends on the hydrolysis of the probe). Melting curves of sequence-specific probes are used for genotyping, resolving single base mismatches between target sequence and probe (Lay and Wittwer 1997; Whitcombe, Brownie et al. 1998), whereas SYBR Green I is used most frequently for product characterization (Ririe, Rasmussen et al. 1997). It has been reported that melting curves measured with SYBR Green I can also be utilized for genotyping of insertion/deletion polymorphisms and of single nucleotide polymorphisms (SNPs) (Akey, Sosnoski et al. 2001; Lin, Tseng et al. 2001).

In melting curves, the signal decreases gradually as a result of a temperature-dependent quench and more abruptly at a certain temperature because of the melting of the products

(dsDNA or ssDNA/probe hybrid). The melting temperature (T_m) of a product is defined as the temperature at which the steepest decrease of signal occurs. This can be identified conveniently as the peak value(s) (global or local maxima) in the negative derivative of the melting curve. Additionally, the area under the curve (AUC) of the peaks is proportional to the amount of product. Therefore, melting curve analysis may be used for quantifications with internal standardization when the T_m values of sample and competitor products are significantly different (Al-Robaiy, Rupf et al. 2001). However, well-performed normalization is required to reduce the systematic error due to the temperature dependent quench. This quench also limits the sensitivity of melting curve analyses. At present, there is only one software package available that can remove the quench effects from the data (Wilhelm, Pingoud et al. 2003).

With SYBR Green I, the amplification of the correct target sequence can be confirmed. In most cases, nonspecific products have different lengths and therefore deviating melting temperatures (Ririe, Rasmussen et al. 1997).Hybridisation probes, molecular beacons and TaqMan probes are used for mutation detection (Lay and Wittwer 1997; Bernard, Ajioka et al. 1998; Bernard and Wittwer 2000), genotyping (Whitcombe, Brownie et al. 1998; Ulvik and Ueland 2001; Grant, Steinlicht et al. 2002; Randen, Sørensen et al. 2003) and SNP screening (Sasvari-Szekely, Gerstner et al. 2000; Mhlanga and Malmberg 2001).

11. Applications

Real-time PCR is used for absolute and relative quantifications of DNA and RNA template molecules and for genotyping in a variety of applications (Wilhelm and Pingoud 2003).

Quantitative real-time PCR is used to determine viral loads (Mackay, Arden et al. 2002),gene expression (Bustin 2000; Goerke, Bayer et al. 2001), titers of germs and contaminations (infood, blood, other body fluids and tissues) (Locatelli, Urso et al. 2000; Hernandez, Rio et al. 2001; Norton 2002), allele imbalances (Ruiz-Ponte, Loidi et al. 2000) and the degrees of amplification and deletion ofgenes (Chiang, Wei et al. 1999; Nigro, Takahashi et al. 2001).

Real-time PCR is also becoming increasingly important in thediagnosis of tumors, such as for the detection and monitoringof minimal residual diseases (Marcucci, Livak et al. 1998; Elmaagacli, Beelen et al. 2000; Amabile, Giannini et al. 2001; Krauter, Heil et al. 2001; Krauter, Hoellge et al. 2001), the identification of micrometastases in colorectal cancer (Bustin, Gyselman et al. 1999), neuroblastoma (Cheung and Cheung 2001) and prostate cancer (Gelmini, Tricarico et al. 2001). It has been used to quantify amplifications of oncogenes (Bieche, Laurendeau et al. 1999; Lehmann, Glöckner et al. 2000; Lyon, Millson et al. 2001; Konigshoff, Wilhelm et al. 2003) as well as deletions of tumor suppressor genes in tumor samples (Wilhelm and Pingoud 2003). Also, the response of human cancer to drugs has been studied (Au, Chim et al. 2002; Miyoshi, Ando et al. 2002;Reimer, Koczan et al. 2002). Other clinically relevant applications are cytokine mRNA profiling in immune response (Hempel, Smith et al. 2002; Stordeur, Poulin et al. 2002) and tissue-specific gene expression analysis (Bustin 2002; Poola 2003; Prieto-Alamo, Cabrera-Luque et al. 2003).

Also, the results of DNA chip experiments are validated by real-time PCR quantifications (Miyazato, Ueno et al. 2001; Rickman, Bobek et al. 2001;Crnogorac-Jurcevic, Efthimiou et al. 2002).

Chimerism analysis is possible when sequence-specific probes are utilized to differentiate and quantify alleles. High dynamic ranges can be achieved with allele-specific real-time PCR (Shively, Chang et al. 2003). Robust chimerism analyses with extremely large dynamic ranges based on insertion/deletion polymorphisms and on SNPs are also possible (Wilhelm, Reuter et al. 2002; Maas, Schaap et al. 2003). Genetic chimerisms have been monitored by Y-chromosome-specific real-time PCR for sex-mismatched transplantations (Fehse, Chukhlovin et al. 2001; Byrne, Huang et al. 2002; Elmaagacli 2002) and by allele-specific real-time PCR (Maas, Schaap et al. 2003; Shively, Chang et al. 2003). This combination of allele-specific amplification with real-time PCR has been shown to reveal detection limits of down to 0.01% for SNPs (Maas, Schaap et al. 2003). Real-time PCR is increasingly used in forensic analyses (Andreasson, Gyllensten et al. 2002; von Wurmb-Schwark, Higuchi et al. 2002; Ye, Parra et al. 2002), but also to monitor disease- or age-related accumulation of deletions in the mitochondrial genome (Mehmet, Ahmed et al. 2001; He, Chinnery et al. 2002).

Melting curve analyses are used for real-time competitive PCR (Al-Robaiy, Rupf et al. 2001; Lyon, Millson et al. 2001), gene dosage tests (Ruiz-Ponte, Loidi et al. 2000) and genotyping and SNP detection (Bullock, Bruns et al. 2002; Burian, Grosch et al. 2002; Randen, Sørensen et al. 2003). These applications will have a particularly strong impact on pharmacogenetics (Palladino, Kay et al. 2003). Profiling of DNA methylation is also possible by melting curve

analysis (Worm, Aggerholm et al. 2001; Akey, Akey et al. 2002), which simplifies the analysis of epigenetic variations of the genome and developmental processes.

In brief, the advantages of real-time PCR are exploited in clinical diagnosis and the monitoring of infectious diseases and tumors. The technique is applied for the analysis of age dependent diseases, cytokine and tissue-specific expression, forensic samples, epigenetic factors like DNA methylation and for food monitoring. The field of applications is still growing rapidly, which suggests that real-time PCR will become one of the most important techniques in molecular life sciences and medicine (Wilhelm and Pingoud 2003).

12. Normalization

Gene expression analysis at the messenger RNA (mRNA) level has become increasingly important in biological research. Generally we detect RNAs to determine if differences protein expression could be explained at the transcriptional level. In particular, measurement of mRNA is needed in situations where quantification of the protein is difficult or cumbersome. Most recently, mRNA expression analysis is being used to provide insight into complex regulatory networks and to identify genes relevant to new biological processes or implicated in diseases (Hendriks-Balk, Michel et al. 2007).Common methods for RNA detection include: Northern blotting, in situ hybridization, qualitative RTPCR, RNase protection assay, competitive RT-PCR, microarray analysis, and quantitative real-time PCR. The specificity, wide dynamic range , ease-of-use , requiring a minimal amount of RNA, no post-PCR handling and avoiding the use of radioactivity, has made the real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) the method of choice for quantitating RNA levels (Radonic, Thulke et al. 2004). The technique has two main steps: CDNA synthesis by reverse transcription of mRNA and subsequent quantification of specific CDNAs by real-time PCR. It is in many cases the only method for measuring mRNA levels of vivo low copy number targets of interest for which alternative assays either do not exist or lack the required sensitivity so these specification has led to made it the "gold standard" for mRNA quantification (Huggett, Dheda et al. 2005). Most gene expression assays are based on the comparison of two or more samples and require uniform sampling conditions for this comparison to be valid. Unfortunately, many factors can contribute to variability in the analysis of samples, making the results difficult to reproduce between experiments. During the preparation of CDNA for real-time PCR analysis there is significant potential for small errors to accumulate. For example, differences in sample size, RNA extraction efficiency, pippetting accuracy and reverse transcription efficiency will all add variability to your samples (Huggett, Dheda et al. 2005). Not only can the quantity and quality of RNA extracted from multiple samples vary, but even replicates can vary dramatically due to factors such as sample degradation, extraction efficiency, and contamination. On the other hand, since many biological samples contain inhibitors of the RT and/or the PCR step, it is crucial to assess the presence of any inhibitors of polymerase activity in RT and PCR. so it is clear that we need to incorporate some normalization method to control for errors. The identification of a valid reference for data normalisation remains the most stubborn of problems and none of the solutions proposed are ideal. Normalization methods range from ensuring that a similar sample size is chosen to using an internal housekeeping or reference gene (Table 3) (Huggett, Dheda et al. 2005).

Normalisation strategy	Pros	Cons	Note
Similar sample size/tissue volume	Relatively easy	Sample size/tissue volume may be difficult to estimate and/or may not be biological representative	Simple first step to reduce experimental error
Total RNA	Ensures similar reverse transcriptase input. May provide information on the integrity (depending on technique used)	Does not control for error introduced at the reverse transcription or PCR stages. Assumes no variation in rRNA/mRNA ratio	Requires a good method of assessing quality and quantity
Genomic DNA	Give an idea of the cellular sample size.	May vary in copy number per cell. Difficult to extract with RNA	Rarely used. Can be measured optically or by real time PCR
Reference genes ribosomal RNAs (rRNA)	Internal control that is subject to the same conditions as the RNA of interest. Also measured by real time RT-PCR	Must be validated using the same experimental samples. Resolution of assay is defined by the error of the reference gene	Oligo dt priming of RNA for reverse transcription will not work well with rRNA as no polyA tail is present. Usually in high abundance
Reference genes messenger RNAs (mRNA)	Internal control that is subject to the same conditions as the mRNA of interest. Also measured by real time RT-PCR	Must be validated using the same experimental samples. Resolution of assay is defined by the error of the reference gene	Most, but not all, of mRNAs contain polyA tails and can be primed with oligo dt for reverse transcription
Alien molecules	Internal control that is subject to most of the conditions as the mRNA of interest. Is without the biological variability of a reference gene	Must be identified and cloned or synthesised. Unlike the RNA of interest, is not extracted from the within the cells	Requires more characterisation and to be made available commercially

There is good correlation between the RNA concentration used and the real time PCR estimation of the different amounts of HuPO cDNA (using omniscript reverse transcriptase).

(Huggett, Dheda et al. 2005)

Table 3. Comparison of the actual amount of RNA used in different reverse transcription reactions with the respective amount of HuPO
Comparison of the actual amount of RNA used in different reverse transcription reactions with the respective amount of HuPO
cDNA measured by real-time RT-PCR

12.1 Methods of normalization

1. Standardizing Sample size

The most basic method of normalization ensures that an experiment compares similar sample sizes and this is achieved by measuring tissue weight, volume or cell number. This method can reduce the experimental error of first stage of qRT-PCR. It seems to be straightforward, but we can't ensure that equal volume of different samples contain the same cellular material. Real-time RT-PCR experiments that rely on the extraction of RNA from complex tissue samples are averaging the data from numerous, variable subpopulations of cells of different lineage at different stages of differentiation (Bustin, Benes et al. 2005). This can be misleading, as is illustrated when sampling a similar volume of blood from HIV +ve patients. Patients with HIV that have less advanced immunosupression (CD4 counts X200 cells/ml) will yield a higher amount RNA than patients with CD4 counts p200 cells/ml. This is simply because there are fewer cells per

milliliter of blood in the latter group. Even cellular subpopulations of the same pathological origin can be highly heterogeneous. Tumor biopsies, in particular, are made up not just of normal and cancer epithelial cells, but there may be several subclones of epithelial cancer cells together with stromal, immune and vascular components (Vandesompele, De Preter et al. 2002; Bustin, Benes et al. 2005). This variability can give us misleading or meaningless result to solve this we can use laser capture microdissection to normalize against the dissected area which can report the target mRNA levels conveniently as copies per area or cell dissected. In in vitro cell culture, due to different morphologies or clumping up of cells, it's hard to determine sample size (cell number) .we can treat them with buffers and/or enzymes till they could be counted, however these treatments surely could affect gene expression. This approach could not be applied for solid tumors for which the amount of cells cannot be determined accurately. To work around this problem, it was suggested to standardize the RT-qPCR data between samples using the amount of genomic DNA as an indicator reflecting the number of cells in each sample. However, these approaches do not account for the degradation of RNA or the efficiency of RT and PCR. So, while ensuring a similar sample size is important it clearly is not sufficient on its own (Huggett, Dheda et al. 2005).

2. Normalization with genomic DNA

Another method for normalization is measuring the amount of genomic DNA (gDNA). This appears to be an ideal method as it does not require reverse transcription for detection by real-time PCR (Bustin 2002). However, this approach do not account for the degradation of RNA or the efficiency of RT and PCR. .Moreover, in the case of normalization with genomic DNA, the fact of working with tumor cells can present additional problems because they tend to have abnormal karyotype. Therefore, the ratio between the amount of DNA and cell number is variable (Huggett, Dheda et al. 2005). Another major problem with using this strategy is that RNA extraction procedures are usually not designed to purify DNA, so the extraction rate may vary between different samples, with DNA yields often being low. In conclude Normalization against genomic DNA is rarely used since it is difficult to coextract with RNA and it may vary in copy number per cell (Huggett, Dheda et al. 2005).

3. Normalization with total RNA

The normalization of RT-qPCR results can be compared to the amount of total RNA used in the reverse transcription step. Not only does this facilitates normalization but circumvents problems associated with the linearity of the reverse transcriptase step. There are several methods for quantifying RNA; the most common is to measure the absorbance at 260 nm (A_{260}) with a UV spectrophotometer. The major advantage of this spectrometer, whose sensitivity is estimated at 5 ng/uL, is that it requires only 1 microL of sample, placed in direct contact with the optical system (Huggett, Dheda et al. 2005; Hendriks-Balk, Michel et al. 2007). However, contaminants absorbing at 260 nm, such as proteins, phenol or genomic DNA, can lead to overestimated results. Another optical system is flourimetry in which intercalating fluorescent nucleic acid is used, the kit RiboGreen ® Molecular Probes based on this principle. This is a more sensitive technique but does not discriminate RNA from DNA, and contaminants such as phenol can produce variable results (Huggett, Dheda et al. 2005). Since it is generally assumed that OD260 analysis is less accurate than the RiboGreen assay, we have compared RNA quantification data obtained using the RiboGreen assay with

OD260 analysis using a Genequant II (Pharmacia). The results (Fig. 2) suggest that both methods generate comparable results when the RNA concentration is not less than 100 ng/µl, with RiboGreen measurements lower than those obtained using the spectrophotometer. OD260 analysis becomes less reliable at lower RNA concentrations (Bustin 2002).
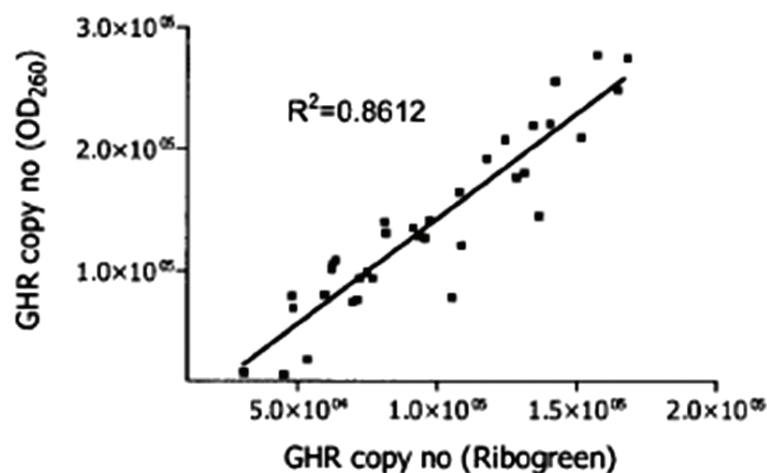

Fig. 2. Comparison of RNA quantification using the Genequant II and the RiboGreen fluorescent assay. RNA from 34 normal colon biopsies was quantitated using a standard Genequant II protocol which measures the absorbance at 260 nm. The same samples were then quantitated using a standard RiboGreen fluorescent assay. RT-PCR assays targeting the GHR were carried out and Ct normalised against the respective concentrations determined by the two methods. The scatterplot shows a good correlation between the two methods ($r2=0.8612$) (Bustin 2002).

What is also important, but often overlooked, is the need to assess the quality of RNA because degraded RNAs may adversely affect results. The opportune development of Agilent's 2100 Bioanalyser and LabChip technology has provided a new standard of RNA quality control as well as permitting concomitant quantification of RNA. The analysis is not influenced by contamination of phenol or proteins, against the presence of genomic DNA requires a correction of the measurement of the concentration of RNA (Bustin, Benes et al. 2005). This is particularly important when the RNA has been extracted from 'dirty' tissue such as the colon (Vandesompele, De Preter et al. 2002). This technique allows characterizing the RNA in a concentration between 5 and 500 ng/uL. For each sample, the software determines the ratio 28S/18S and assigns a RIN (RNA Integrity Number) which takes into account the entire electropherogram. The value of RIN ranges from 1 to 10, with 1 being totally degraded RNA, and 10 to a high-quality RNA. In addition to being fast and allow high throughput, this technique requires only 1 microL of sample. It is the simplest method and objective qualitative analysis of RNA, its use is recommended (Schmittgen and Zakrajsek 2000; Bustin 2002). Similar in concept, but requiring an additional RT-PCR assay, is normalization against one of the rRNAs. rRNA levels may vary less under conditions that affect the expression of mRNAs and the use of rRNA has been claimed to be more reliable than that of several reference genes in rat livers and human skin fibroblasts (Bustin 2002; Huggett, Dheda et al. 2005). But, a drawback is that it primarily measures ribosomal RNA (rRNA) whereas real-time PCR aims to determine mRNA expression and normalization for

total RNA assumes that the rRNA:mRNA ratio is the same in all groups, which might not always be the case. Moreover, rRNA is not present in purified mRNA and the high abundance of rRNA compared to mRNA makes it difficult to subtract the baseline value in realtime PCR analysis. Thus, markers of rRNA such as 18S or 28S rRNA might also be suboptimal as normalization factors in many settings (Hendriks-Balk, Michel et al. 2007). Also, it has been reported that rRNA transcription is affected by biological factors and drugs. An important parameter to consider when normalized relative to the RNA is the quality of it. Differences in the quality of the samples strongly depend on the extraction step and are the source of the most common variations in RT-qPCR. It is therefore important to use the same method of extraction for all samples analyzed (Vandesompele, De Preter et al. 2002). The quality of RNA is defined by both its purity (no contamination) and integrity (non-degraded RNA). Its purity was determined by measuring the absorbance at 230 (organic contaminants) and 280 nm (specific proteins), RNA is considered pure ratios A_{260}/A_{230} and $A_{260} / A_{280} > 1.8$. With regard to the integrity, the traditional method was to visualize the bands of 28S and 18S ribosomal RNA on a gel electrophoresis. Indeed, it is difficult to analyze directly the mRNA; they represent only 1% to 3% of total RNA. We must therefore consider that the degradation of ribosomal RNA, the majority, reflecting the degradation of mRNA. Thus, the 18S/28S ratio assesses the integrity of RNA; a ratio close to 2 is considered an indicator of RNA with little or no gradient. However, this method requires a large amount of RNA (0.5-2 mg), and is not sensitive enough to detect slight damage. Normalizing a sample against total RNA has the drawback of not controlling for variation inherent in the reverse transcription or PCR reactions and it ignores the efficiency of converting RNA into CDNA. Also rRNA cannot be used for normalization when quantifying targets from polyA-enriched samples (Huggett, Dheda et al. 2005). A final drawback when using total RNA for normalization is the lack of internal control for RT or PCR inhibitors. All quantitative methods assume that the RNA targets are reverse transcribed and subsequently amplified with similar efficiency but the reaction is extremely sensitive to the presence of inhibitors, which can be reagents used in the extraction step (salts, alcohols, phenols), or components copurification organic (urea, heme, heparin, immunoglobulin G). These compounds can also inhibit the PCR reaction. Thus, two reactions with an equal amount of RNA, but the efficiencies of RT and / or PCR are different, will yield results that cannot be compared. Different methods exist to assess the presence of inhibitors in biological samples. First, it is possible to compare the efficacy of PCR for different dilutions (1/20 and 1/80 for example) of a sample. An alternative is to add a defined amount of a synthetic single-stranded amplicon CDNA samples, and comparing its amplification compared to a control without CDNA. However, these methods are limited to verify the absence of PCR inhibitors, and do not evaluate the effectiveness of the reverse transcription step (Bustin 2002).

4. Normalization with an artificial molecule (spike)

An interesting solution to control the two enzymatic reactions (RT and PCR) is added to RNA extracted an exogenous RNA, which will compare the amplification between the different samples. This sequence control should show no similarity to the target RNA, we will use such a specific mRNA from a plant when studying gene expression in humans. The main criticism of using spikes is that, while they can be introduced prior to extraction, unlike the cellular RNAs they are not extracted from within the tissue. Consequently, there

may be situations (e.g. if the samples differ histologically) when the spike may not be a good control for the extraction procedure. The stages required to generate the alien molecule may also not be feasible for small laboratories wanting to perform limited amounts of real-time RTPCR (Schmittgen and Zakrajsek 2000; Argyropoulos, Psallida et al. 2006).

5. Normalization with reference genes

Reference genes represent the by far most common method for normalizing qRT-PCR data. Reference genes are often referred to as housekeeping genes assuming that those genes are expressed at a constant level in various tissues at all stages of development and are unaffected by the experimental treatment (Hendriks-Balk, Michel et al. 2007; Balogh, Paragh et al. 2008). Use of this endogenous control theory allows controlling all stages of the experimental protocol; its expression reflects not only the quantity and quality of RNA used, but the efficiencies of the RT and PCR. An advantage of reference genes as compared to total or rRNA is that the reference gene is subject to the same conditions as the mRNA of interest (Hendriks-Balk, Michel et al. 2007). The most commonly used reference genes include β-actin (ACTB), (GAPDH), (HPRT) and 18S rRNA. The other commonly used reference genes would be PGK1, B2M, GAPD, HMBS, HPRT1, RPL13A, SDHA, TBP, UBC and YWHAZ (Vandesompele, De Preter et al. 2002). The initial concentration of a target is usually derived from the C_T (cycle threshold), which is the number of amplification cycles where the amplification curve crosses the threshold line. This line is placed at the exponential phase, so as to be clearly distinguishable from background noise. For each sample, the C_T obtained for the genes of interest and reference must be converted to normalized expression ratio. For this, various options are available, they are integrated in the software provided with the various qPCR instruments or described in the literature. The relative standard curve method requires the construction, for the target gene and reference gene, a range made from a series of dilutions of a reference sample. These ranges to obtain standard curves, obtained by expressing the C_T as a function of log of the initial concentration of cDNA. Concentration values for each point of the range can be set arbitrarily in accordance with the dilution factors. Therefore, the relative amount of a target is determined by the C_T by interpolation with the standard curve. The standard expression of a gene of interest is determined by the following formula:

$$R = \frac{Relative\ amount\ of\ the\ gene\ of\ intrest}{Relative\ amount\ of\ the\ refrence\ gene}$$

In addition, a calibrator is typically used. This is a sample used as a basis for comparing results. The normalized ratio of each sample is divided by the normalized ratio of the calibrator. Thus, the calibrator becomes the reference 1x, and all other samples are expressed as a ratio relative to the calibrator. The method of $\Delta\Delta C_T$ uses a mathematical formule to calculate the ratio of expression of a target gene between two samples, normalized with reference gene. First, the differences ΔC_T between the values of C_T target gene and reference gene were determined for the test sample and control.

$$\Delta C_{T\ (sample)} = C_T\ (target\ sample) - C_T\ (reference\ sample)$$

$$\Delta C_{t\ (control)} = C_T\ (target\ control) - C_T\ (reference\ control)$$

Next, the $\Delta\Delta C_T$ between control and the sample is calculated:

$$\Delta\Delta C_T \, \Delta C_{T \, (control)} - \Delta C_{T \, (sample)}$$

Finally, the normalized ratio of expression of a target gene is determined by the formula: $2^{-\Delta\Delta CT}$.

Unlike the relative standard curve method, where the amplification efficiency (E) target genes and reference is directly taken into account when building ranges, the method of $\Delta\Delta C_T$ is assumed that the efficiencies of the two genes are equal to 100% (E = 2, with each cycle of the exponential phase, the concentration of PCR products is doubled). However, a difference in PCR efficiency of 3% ($\Delta E = 0.03$) between the two genes results in an error of 47% for the ratio of expression if $E_{target} < E_{ref}$ and 209% if $E_{target} > E_{ref}$ after 25 cycles. In addition, the error increases exponentially with larger variations of efficiency and a greater number of cycles. New models have been developed taking into account the efficiency of PCR target gene and reference gene. The most common is the model of Pfaffl, where the relative expression ratio (R) of a target gene between a sample and control is determined by the following formula:

$$R = \frac{(E_{target})^{\Delta C\,T_{target}(control\,-sample)}}{(E_{reference})^{\Delta C\,T_{reference}(control\,-sample)}}$$

In this model of Pfaffl, the efficiency of PCR for a given gene is calculated from the construction of a calibration curve using the following formula: $E = 10^{[-1/\,gradient]}$. This method gives a good estimate of effectiveness, although it is possible that it is overestimated. However, this approach assumes that the amplification efficiencies between the diluted samples are identical, creating a linear relationship between C_T and amount of CDNA in the beginning. Therefore, some authors such as Liu and Saint have developed models that take into account standards of efficiency for each sample, the latter being determined by the kinetics of the amplification curve. However, with this kind of approach, the slightest error in the measurement of effectiveness is amplified and passed exponentially on the expression ratio calculated. The different models of normalization with reference genes therefore have all the advantages and disadvantages. At present, there is no time-honored method for the treatment of the results of RT-qPCR. Normalization to a reference gene is a simple method and frequently used because it can control many variables. An advantage of reference genes as compared to total or rRNA is that the reference gene is subject to the same conditions as the mRNA of interest (Bustin, Benes et al. 2005; Hendriks-Balk, Michel et al. 2007). What has become apparent over recent years is that there is no single reference gene for all experimental systems. Quantified errors related to the use of a single reference gene as more than three-fold in 25% and more than six-fold in 10% of samples. Today it is clear that reference genes must be carefully validated for each experimental situation and those new experimental conditions or different tissue samples require re-validation of the chosen reference genes (Balogh, Paragh et al. 2008). If inappropriate reference genes are used for normalization, the experimental results obtained can differ greatly from those using a validated reference gene. Validation of a reference gene requires removal of any non-specific variation in expression. This can be done using a

recently introduced program called geNorm (freely available at http://medgen.ugent.be/~jvdesomp/genorm/) that mathematically identifies the most suitable reference gene for a given experimental condition. Because of the inherent variation in the expression of reference genes the use of multiple reference genes rather than one reference gene is recommended to ensure reliable normalization of real-time PCR. Several statistical programs can help to determine the most appropriate reference gene or set of genes (Hendriks-Balk, Michel et al. 2007; Borges, Ferreira et al. 2010).

13. Abbreviation

ACTB: Beta actin
B2M: Beta-2-microglobulin
C_T: Threshold Cycle
FRET: Fluorescence Resonance Energy Transfer
GAPD: Glyceraldehyde-3- phosphate dehydrogenase
HMBS: Hydroxymethyl-bilane synthase
HPRT: hypoxanthine ribosyltransferase
HPRT1: Hypoxanthine phosphoribosyl-transferase 1
HRM: High Resolution Melting
KPCR: Kinetic Polymerase Chain Reaction
LNA: Locked Nucleic Acid
LUX: Light Upon Extension
PCR: Polymerase Chain Reaction
PGK1: phosphoglycerokinase 1
PNA: Peptide Nucleic Acid
Q-PCR (QRT-PCR): Quantitative Real-Time Polymerase Chain Reaction
RPL13A: Ribosomal protein L13a
RT PCR: Reverse transcription PCR
SDHA: Succinate dehydrogenase complex, subunit A
TBP: TATA box binding protein
T_m: Melting Point
UBC: Ubiquitin C
YWHAZ: Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide

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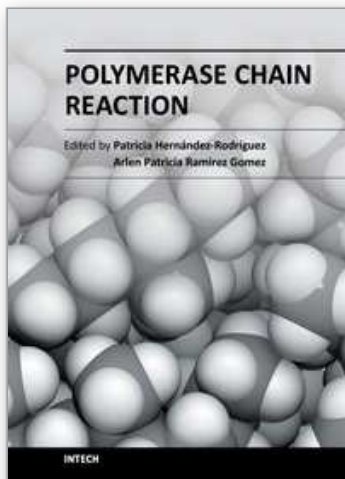
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