Real-Time PCR Analysis of Cardiac Samples

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Introduction

A proper understanding of the workings of the heart requires an appreciation of the engines that make cardiac excitation and contraction possible. These ion channels, pumps and exchangers are all central to this process, and are in turn regulated by many other proteins which assist them in governing membrane current flow. Further regulation is provided by the autonomic nervous system, which exerts a profound effect on cardiac function as a whole by controlling the activity of proteins that affect both heart rate and contractility. Pathologies often arise as a result of aberrant or inappropriate protein expression and the subsequent destruction of equilibrium. As such, the profiling of protein expressions in both diseased and normal states can yield important clues as to how diseases evolve. Moreover, it can allow researchers to identify proteins critical to the development of certain pathologies, and target them for pharmacological intervention. A first step in this process can be the careful study and comparison of the RNA expression profiles of diseased and normal hearts. Comparing RNA levels is often used, as the expression of a given protein often follows closely the expression of its RNA.

The methods available for analyzing mRNA expression levels are diverse. The factors used when deciding upon the best approach are equally varied and depend in part upon the type of data the researcher is seeking. The type of sample material available, the quantity of the material, and technical proficiency of the lab must all be taken into consideration. Northern blotting, *in situ* hybridization, RNase protection assays, cDNA arrays and RT-PCR are all tried and true techniques when comparative data are sought. For the researcher requiring data that is more exacting, quantitative PCR is the method of choice. There have been three basic incarnations of "quantitative" PCR to date: Semi-quantitative PCR, competitive quantitative PCR and Real-Time PCR (Bustin 2000; 2002; Ong and Irvine 2002; Overbergh et al. 2003). What is the advantage of performing quantitative PCR vs. normal PCR in acquiring quantitative data? To answer this question one must first appreciate the limitations inherent in classical polymerase chain reactions.

During the initial phase of a PCR reaction, the concentration of reagents i.e. the primers, and dNTP's is in great excess. Conversely, the concentration of template and amplicons is relatively low. This small amount of product helps to drive the reaction forward, eventually leading to the accumulation of amplicons at an exponential rate.

At a certain point, the rate of product formation plateaus and becomes minimal. Exactly when this happens for a given primer template combination is extremely variable, but we do know that it occurs when the accumulated product effectively becomes a feedback inhibitor of the very reaction that produces it. More specifically, product begins to preferentially re-anneal with itself as opposed to primer, thereby causing the production of new amplicons to slow. This is typically the phase many PCR reactions are in when they are halted and the samples removed for analysis. Yet, because the reaction is no longer exponential, it is the least likely point at which to obtain credible quantitative data. This is because analysis of samples during the exponential phase, results in quantitative data that is extremely reproducible, and provides the researchers with several orders of magnitude of dynamic range over which to analyze the data. Only in the exponential phase can the amount of gene expression be made readily available to the researcher, and as a result the overall relevance of a given gene in the expression profile for a specific pathology be assessed. The three basic types of quantitative PCR address this challenge of acquiring data from the exponential phase by using different approaches.

Semi-quantitative RT-PCR is the least sophisticated of the three techniques mentioned. In semi-quantitative PCR, a researcher monitors product accumulation during the exponential phase, by interrupting the amplification at a cycle that was predetermined, as a result of an optimization process that is sometimes laborious. More specifically, the exponential phase for a given reaction is determined, by halting PCR reactions at the end of each cycle and assessing the amount of product formed. While useful, semi-quantitative RT-PCR is limited by a relatively small dynamic range,

Quantitative-competitive PCR obtains quantitative data by a different means. It involves the simultaneous amplification of both the target and a competitor (internal standard) in the same tube. This internal standard is usually DNA, which can be amplified with primers specific for the target sequence, but can be distinguished from the target by size or internal sequence. The amount of target template is, therefore, quantified by the titration of an unknown amount of target template against a dilution series of known amounts of the competitor sequence. Quantitative-competitive PCR is limited by the inherent difficulty in designing a functional competitor, as well as the labor involved in optimizing the technique. Additionally, there are no guarantees that absolute quantification will be achievable using the internal standard, because differences in the amplification efficiencies between the control and target DNA can go undetected.

The post-amplification steps required to detect PCR products, further complicate acquiring quantitative data from either semi-quantitative or quantitative-competitive PCR. Ethidium bromide staining of agarose gels, the use of fluorescence labeling, radioactive labeling, southern blotting etc. all require numerous steps which are both laborious and time consuming. Many of the techniques also pose hazards to both the researcher and the environment, whether from toxic molecules or radiochemicals. Additionally, these techniques require imaging the result and quantifying the image. This is a significant source of error in obtaining a quantification of the result.

Quantitative Real-Time PCR manages to overcome all of the troubles associated with the pursuit of quantitative data. Real-Time PCR allows for the procurement of quantitative data during the critical exponential phase of amplification reaction, with-

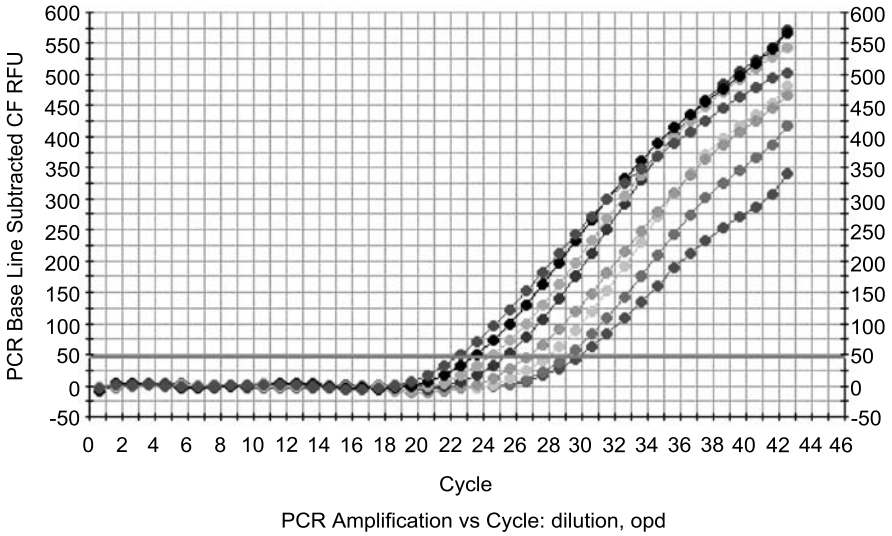

Figure 1
Example of a Real-Time PCR Result: A sample result was extracted from the iCycler software tutorial. Multiple dilutions of primers were used to create a set of values. The Ct (threshold cycle) increases with greater dilution. *For coloured version see appendix*

out requiring the optimization steps required of semi- and competitive-quantitative PCR, or the post-amplification steps required for both methods in order to visualize the PCR products. Real-Time PCR is, therefore, the method of choice because it provides for speed, a reduced risk of contamination and the acquisition of quantitative data.

Just what is Real-Time PCR? Real-Time PCR is based on the detection and quantification of signal generated by a fluorescent reporter, the intensity of which is designed to increase in direct proportion to the amount of PCR product yielded during amplification. The amount of fluorescence produced at each cycle or the delta Rn is derived from the equation Rn+ (fluorescence emission of PCR product at each cycle) – Rn– (the fluorescence emission of the baseline). The delta Rn collected at each cycle is then used to construct amplification plots. The fluorescence emissions data are plotted versus cycle number.

During the early phase of the reaction, the delta Rn values do not exceed threshold, which is arbitrarily set at ten times the standard deviation of the baseline set from cycles 3–15. The resulting curve (Fig. 1), allows for the determination of what is know as the threshold cycle (C_T) or the cycle number at which the amount of fluorescence generated by PCR products becomes detectable above background. By using both a threshold cycle and a standard curve, quantitative information can be generated. The relative levels of fluorescence produced can be accessed while the reaction progresses in real-time. Most important, are the levels recorded during the exponential phase, which is then correlated with the initial amount of template in the reaction tubes.

Description of Methods and Practical Approach

Chemistries

The fluorescence-based methods developed to detect the accumulation of PCR products can be either nonspecific or sequence specific. Nonspecific detection methods make use of fluorescent dsDNA intercalating dyes such as SYBR® green and ethidium bromide. Additionally, they are the simplest and most cost effective methods available. Ethidium bromide helped demonstrate the feasibility of monitoring reactions as they progress in real time. Higuchi et al (Higuchi et al. 1993), using ethidium bromide and a CCD camera, introduced the world to the concept of real time quantitative PCR. Due to ethidium bromide's hazardous nature, a less non-toxic alternative SYBR green is in use by most laboratories today. SYBR® green has an additional benefit of fluorescing only when incorporated into double stranded DNA (Fig. 2). The greatest advantage of a non-specific detection system is its flexibility. SYBR green can be used with any primer pair to amplify any target. Therefore it is a very cost effective and safe way to generate quantitative data. This flexibility, however, is a double-edged sword. SYBR green lacks sequence specificity, and it will detect non-specific PCR products and primer-dimers as well as the legitimate amplicons. This can result in the overestimation of the target DNA concentration. A combination of techniques can be used to circumvent this drawback. Firstly, melt curve analysis should be performed, enabling the differentiation of fluorescence originating from target DNA and that produced by primer-dimers or spurious PCR products. Additionally, careful design of primers and reaction optimization are fundamental to the proper function of any PCR reaction, and can also reduce non-specific fluorescence. Lastly, a Hot-Start protocol should be employed to minimize the amplification of illegitimate PCR products.

Specific detection methods rely on the principle of Fluorescence Resonance Energy Transfer or FRET, which is based on the transfer of energy from a donor molecule to an acceptor molecule. The degree of energy transfer is directly proportional to the distance between the moieties. In the case of two fluorophores with overlapping excitation and emission spectra, excitation of the donor molecule will result in a corresponding increase in fluorescence from the acceptor molecule, if the two molecules are in close enough proximity. When this principle is applied for sequence specific

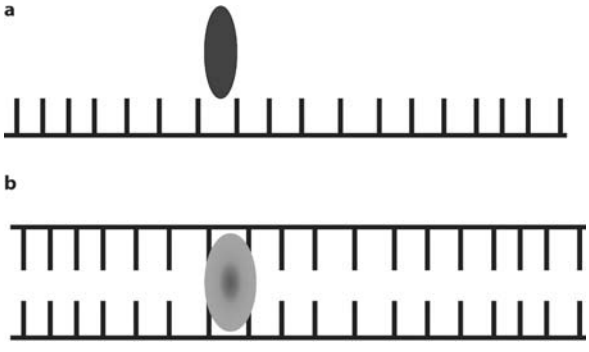

Figure 2a,b
Illustration of the SYBR green reaction: **a** SYBR green does not fluoresce when free or associated with single stranded DNA. **b** SYBER green fluorescens when bound to double-stranded DNA. *For coloured version see appendix*

detection in Real-Time assays, donor and acceptor molecules are placed at either side of a probe complimentary to the target sequence. The technique has been further modified by the replacement of the fluorogenic acceptor with a non-fluorogenic acceptor molecule or quencher. Therefore, when the donor and acceptor molecules are in close proximity, no fluorescence will be detected. However, when the distance between the two is increased, the reporter dye will fluoresce unimpeded. Fluorescent reporter dyes commonly used include FAM (6-carboxyfluorescein), TET (tetrachloro-6-carboxyfluorescein), Joe (2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein), HEX (hexachloro-6-carboxyfluorescein) and VIC. DABCYL and TAMRA are both popular quenchers, and have been used widely and effectively in Real-Time PCR applications. These quenchers, however, suffer from well-known individual weaknesses, such as the poor excitation and emissions overlap of DABCYL with the fluorescent dyes, or the auto fluorescence of TAMRA. New and improved quenchers called "Black Hole Quenchers" have been developed and are being marketed. Black Hole quenchers unlike DABCYL can quench over the entire range of the visible spectrum and unlike TAMRA do not autofluoresce.

Two specific detection methods, "Molecular Beacons" and "Scorpion primers", achieve separation between reporter and quencher dyes by hybridization with target DNA during the annealing phase of the amplification cycle. TaqMan assays, on the other hand, achieve separation through the enzymatic cleavage of the probes during the extension phase of the PCR reaction. Regardless of the method or technique used, FRET allows for the real-time monitoring and quantification of PCR product accumulation in a sequence specific manner.

TaqMan probes are based on 5' nuclease assay technology. TaqMan probes are comprised of a target sequence specific probe labeled at the 5' end, with a fluorescent reporter dye and at the 3' end with a quencher. Little, or no fluorescence, is emitted by the TaqMan probe, in either the hybridized or unhybridized states, due to the closeness of the reporter and quencher moieties. Hybridization of probe to target sequence during PCR renders the probe accessible to the 5'-3' exonuclease activity of Taq polymerase. The degradation of the probe results in the separation of the fluorophore from the quencher, allowing it to fluoresce freely (Fig. 3). Therefore, as more PCR product is generated, more probe are degraded, and the level of fluorescence increases proportionally.

Molecular Beacons (Marras et al. 1999; Tyagi et al. 1998; Tyagi and Kramer 1996) like TaqMan probes, possess a reporter dye at the 5' end and a quencher at the other. Unlike TaqMan probes, Molecular Beacons adopt a stem-loop configuration to bring the reporter and quencher in close proximity, which facilitates FRET. Fluorescence of the reporter dye is achieved when the probe anneals with its target sequence and is in an extended configuration. The increased distance between reporter and quencher prevents FRET from occurring, allowing the accumulation of PCR product to be monitored as an increase in fluorescent emissions from the hybridized probe. Molecular Beacons have a higher specificity than TaqMan probes, which is partly due to the stem loop structure they adopt in the unhybridized state (Fig. 4). They are, therefore, especially useful for SNP analysis, as they can distinguish between targets differing by a single nucleotide and have been employed extensively in the area of mutation detection.

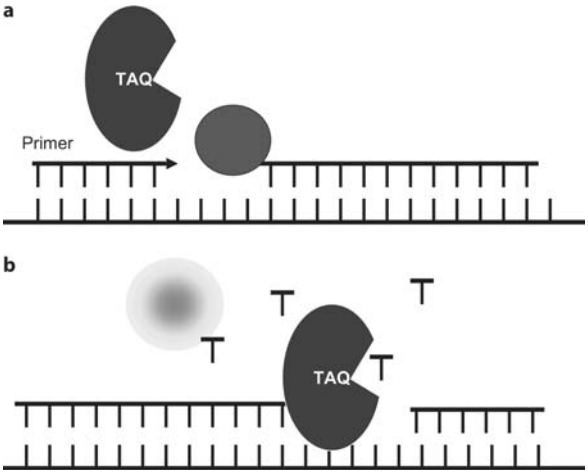

Figure 3a,b
Example of a TaqMan Reaction: **a** Taqman probes allow energy transfer from the fluorescent emitter and the quencher when intact. **b** Taq polymerase has exonuclease activity that digests the probe allowing the emitter to freely fluorescence.*For coloured version see appendix*

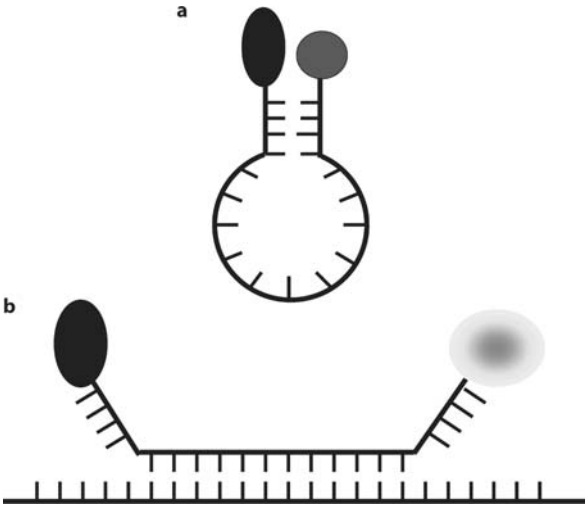

Figure 4a,b
Example of a Molecular Beacon: **a** The native structure of free Molecular Beacon probes brings the emitter and quencher close together inhibiting fluorescence. **b** When product is available, the probe binds to the target, forcing the emitter to separate from the quencher allowing the emitter to freely fluorescence. *For coloured version see appendix*

Scorpion primers (Taveau et al. 2002; Thelwell et al. 2000) represent the next step in the evolution of sequence detection methods. They improve sequence specificity and increase the speed and efficiency of Real-Time PCR assays. Scorpion primers are comprised essentially of a Molecular Beacon with a sequence specific primer attached at the 3' end of the stem-loop configuration. The primers contain a modification called a PCR stopper (typically hexethylene glycol) at their 5' end, which unites the beacon and primer, and prevents the PCR reaction from copying the step loop portion of the Scorpion primer. The molecular beacon portion of the Scorpion primer is designed to bind complimentarily with the extension products of the primer to which it is attached. During the annealing phase of the PCR cycle, the probe sequence curls back to hybridize the target sequence in the amplicon (Fig. 5). This results in the se-

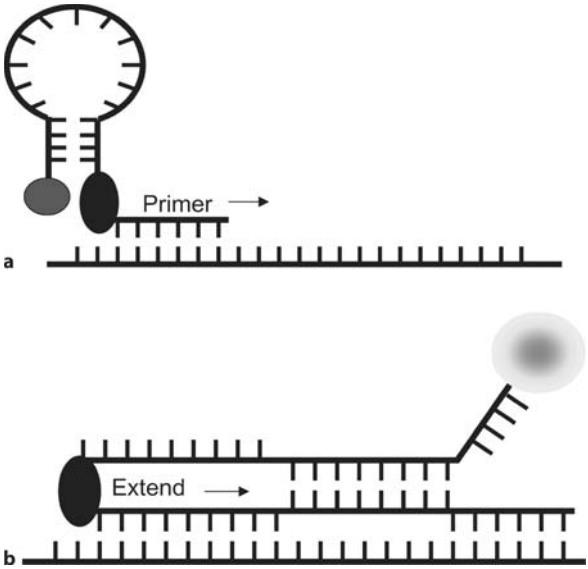

Figure 5a,b
Scorpion Primers. Scorpion primers are able to act as a combination of a PCR primer and Molecular Beacon. **a** The native structure of the scorpion probe brings the emitter and quencher close together inhibiting fluorescence, whilst the other end of the molecule can act as a PCR primer. **b** After extension of a PCR product, the probe binds to the target forcing the emitter to separate from the quencher, allowing the emitter to freely fluoresce. The target is the product of extension of the primer end of the molecule making the detection intra-molecular. *For coloured version see appendix*

paration of the fluorescent dye and quencher at either end of the stem loop configuration, preventing FRET, and allowing for increases in fluorescence in a sequence specific manner.

The uni-molecular configuration of Scorpion primers imbues them with a kinetic advantage over TaqMan and Molecular Beacons because probe-target interaction becomes an intra-molecular event. Therefore, unlike TaqMan and Molecular Beacon assays, fluorescent signal generation is no longer dependent upon the chance meeting of a probe, normally present in low concentrations, with its target. This allows for the use of more rapid cycling conditions, and more importantly, the production of greater signal strengths, as more probe is able to anneal with its corresponding target per given cycle. Scorpion primers possess an additional advantage over TaqMan assays, which they share with Molecular Beacons; PCR reactions using Scorpion primers are performed at the optimal temperature for polymerase activity, whereas TaqMan assays are performed at lower temperatures, which allows for the 5' nuclease activity of the enzyme to function. Scorpion primers have been further tailored to overcome some quenching of reporter dye fluorescence that may occur even in the open configuration. Duplex Scorpion primers achieve greater signal strength by placing dye and quencher on separate complimentary oligonucleotides. The primer, PCR stopper, and probe comprise one half of the duplex Scorpion probe. The other half consists of an oligonucleotide complimentary to the probe sequence with a quencher linked to its 3' end. As with regular Scorpion primers, duplex Scorpion primers retain the kinetic advantages of intra-molecular binding of probe to its target following denaturation and polymerization. During the PCR cycle annealing phase, interaction between probe and primer extension products is favored kinetically over probe-quencher oligonucleotide binding. As a result, greater distances between fluorophore and quencher at the time of signal detection are achieved, thus yielding significantly greater signal in-

tensity with lower background fluorescence levels when compared to the normal Scorpion primer format. Additionally, duplex Scorpion primers are much easier to design and synthesize, as each oligonucleotide requires only one dye be attached, and neither requires the engineering of a stem-loop structure duplex. This significantly reduces the labor required to design these probes, and the effort required to produce them.

Hybridization probes are one of the more complicated applications designed for Real-Time PCR. For this system a total of four oligonucleotides are required: two primers and two probes. Each probe is labeled with either a donor or acceptor dye, and both probes are designed to hybridize to the target in a head to tail manner. This brings the donor and acceptors in close proximity allowing FRET to occur. Unlike Molecular Beacons, TaqMan assays or Scorpion primers, hybridization probes do not utilize a quencher, therefore, the signal produced from the acceptor dye is what is used to track the amount of DNA produced during the PCR reaction.

Peptide nucleic acid (PNA) oligomers have also been used as probes for real-time PCR assays. They are similar in structure to DNA in that they possess a repeating N-(2-aminoethyl)-glycine backbone which mimics the phosphate backbone of DNA. They make excellent highly specific probes because they are non-charged, and therefore require no salt in order to stabilize the duplexes that can form with either RNA or DNA. The melting temperature of PNA/ DNA or PNA/RNA duplexes is, therefore, much higher than those formed by DNA/DNA interactions. This allows duplex formation to occur at low salt conditions while preserving high specificity. The higher melting temperature of PNA's, permits design to be shorter if need be, as a 15-mer PNA probe will possess a similar melting point as a DNA 25-mer. However, specificity will be diminished as probe length decreases, and this must be kept in mind when designing PNA probes.

Instrumentation

Presently there exists a wide variety of instrumentation designed for Real-Time PCR from which to choose. They all overcome the inherent deficiencies in conventional-reverse transcription-PCR, and are capable of monitoring increases in fluorescence during a reaction in real-time. They all attempt to reduce the cost associated with real-time by minimizing the amount of reagent and template needed. The machines will vary primarily in terms of cost, the light source used to stimulate the fluorophores, the amount of samples which can be processed during a typical run, multiplexing capabilities, and the overall friendliness of the user interface.

The ABI 7700 from Applied Biosystems is one of the better known Real-Time PCR machines, primarily because it was the first to become commercially available. Applied Biosystems has followed up the ABI 7700 with the Gene Amp 5700 and the 7900HT, which differs from the ABI 7700, in that up to 384 samples can be processed at a time. Although old in comparison to many other machines, the ABI 7700 remains a viable choice for many researchers. It can be used for 5' nuclease assays (TaqMan), Molecular Beacon assays, as well as non-specific sequence detection methods (SYBR green). The ABI 7700 and the ABI 7900HT are limited perhaps only by the light

sources which they use to excite fluorescent dyes. Both instruments rely on a single excitation laser as a light source, which exposes the two weaknesses of the ABI 7700 and the ABI 7900HT. With a limited excitation range of 488-514, there are fewer dyes available from which the researcher can choose. Additionally, the laser light source, while intense, provides less uniform excitation of fluorophores over a given range. This limitation becomes important when multiplexing or amplifying more than one target in the same tube, and monitoring both reactions via fluorescent emissions from probes labeled with different fluorophores. Therefore, when using the ABI 7700 or ABI 7900HT for multiplexing, it is critical that fluorophores with a dissimilar absorbance and emission spectra be chosen. In the Gene Amp 5700, the excitation laser is replaced with a halogen lamp, which provides uniform excitation over a much broader spectrum of wavelengths. The 5700 does retain the flexibility of the 7700 in terms of the sequence detection chemistries which can be used, but it falls short in that it can only detect one single wavelength, and is therefore incapable of multiplexing.

The LightCycler from Roche Molecular Biochemical's has a very unique approach to Real-Time PCR both in terms of light source, sample containers and thermocycling. It is a very compact machine and utilizes borosilicate capillary tubes to accommodate samples. A blue-light emitting diode is used as a light source, and the resulting emissions are interpreted by three silicon photodiodes that can filter out various wavelengths, which allows the researcher to perform multiplexing reaction with the LightCycler. For thermocycling, air is used as the heating medium, which along with the high surface area to volume ratio of the borosilicate tubes, means that a PCR run of 30–40 cycles can be performed in 20–30 min compared to 2 h. Apart from the obvious advantage of speed, the LightCycler is relatively inexpensive when compared with other machines. Its disadvantages include the required use of capillary tubes as opposed to regular PCR tubes. Additionally, its speed is somewhat negated by the small sample format, in that only 32 reactions can be performed at a given time. This deficiency limits the scope of studies in which a large number of samples must be compared during a single run.

The Biorad iCycler, like the LightCycler, is also a very compact real-time PCR machine. It is a modular machine in that the thermocycler module is not permanently attached to the optics component. They can be and are sold separately. The iCycler uses a halogen tungsten lamp and can amplify up to 96 samples at once. The sample capacity of the iCycler can be extended up to 384 samples for those requiring additional throughput.

The Rotor-Gene from Corbett Research, like the LightCycler, represents a radical departure from other Real-Time PCR machines in terms of design. The Rotor-Gene employs LED light sources, four in number, to excite the fluorescent dyes. Subsequent emissions are monitored via six filters and photomultipliers. The most unique element of the Rotor-Gene is that the real-time reactions are carried out in PCR tubes placed in a 36 or 72 well rotor that spins at 500 rpm during the run. The centrifugal motion is said to reduce temperature equilibration times, and sample-to-sample nonuniformity, thereby improving consistency and reliability.

The Smart Cycler is a recent entry into the Real-Time PCR machine marketplace. It is manufactured by Cephid, and can use TaqMan, Molecular Beacons, Scorpion primers, hybridization probes as well SYBR® green I. It is uniquely flexible, in that

each of its 16 sample wells can be operated independently. This means that up to 16 separate reactions, requiring different thermocycling parameters or optical settings, can be performed simultaneously. The major disadvantage of the Smart Cycler system is the limited sample size format, which may be inadequate for quantitative studies.

The Stratagene MX4000 is also a relatively new option available to researchers in the market for a Real-Time PCR machine. The MX4000 uses a quartz-tungsten halogen lamp and can be used with all of the sequence detection chemistries detailed earlier in this discussion. Its 96 well block can accommodate samples in many formats i.e. tubes or plates. The MX4000 is sold with an integrated personal computer which functions and stores data separately from the Real-Time PCR machine itself, thereby providing protection from possible loss of data if the PCR machine is damaged or disabled.

Example

To determine the utility of Real-Time PCR, we designed a Molecular Beacon probe and primers for use in the Biorad I-cycler. A specific probe for the Kir 2.1 potassium channel was designed and dually labeled with the reporter dye FAM at the 5' end, and the Black Hole Quencher at the 3' end (International DNA Technologies, IDT). For quantification, a standard curve was constructed with Kir 2.1 amplicons that was generated by the primers designed to be used in combination with the probe. A commercially available TET-labeled Cylcophilin probe and primer set (Amersham) was chosen as the internal control for correcting variations in RNA input, and inefficiencies in reverse transcription.

RNA Extraction

There are many choices of systems for isolation of RNA from mammalian tissue. Our own experience has been with TRI Reagent (Molecular Research Center, Inc). This is one of a group of reagents that are composed of a mono-phase mixture of phenol and guanidine thiocyanate (Chadderton et al. 1997; Mannhalter et al. 2000). TRI Reagent is sometimes marketed as TRIzol, an alternative formulation is sold as RNAzol or RNA-Bee by Iso-Tex Diagnostics, Inc. Although we have no experience with this product it should be similar to TRI reagent in its effectiveness. Both reagents utilize phenol and guanidine thiocyanate to inhibit RNAse and denature proteins. Chloroform or an alternative reagent, bromo-chloro-propane (BCP) is then added to separate the pha-ses. RNA is isolated from the upper (aqueous) phase by alcohol precipitation. RNA is quantified spectrophotometrically.

Specific protocol for the isolation of RNA from heart tissue:

1. Dissect and weigh 300 mg of heart tissue
2. Add 3 ml of TRI Reagent (Molecular Research Center, Inc.)
3. Homogenize for 10 s, put on ice for 2 min, repeat 2× more

4. Store the homogenate at room temperature for 5 min
5. Add 0.3 ml of BCP, shake vigorously for 15 s
6. Store sample at room temperature for 10 min
7. Centrifuge at 12,000 *g* for 15 min
8. Transfer upper aqueous phase to new tube
9. Add equal volume of isopropanol, mix and store at room temperature for 10 min
10. Centrifuge at 12,000 *g* for 8 min at 4 °C
11. Remove the supernatant and wash RNA pellet by adding 3 ml of 75% ethanol and centrifuge at 7,500 *g* for 5 min at 4 °C
12. Remove the ethanol and air-dried pellet for 5 min
13. Dissolve RNA in DEPC-water

cDNA Synthesis

There are numerous reverse transcription systems to choose from. These systems vary in their primers, enzyme, as well as other reagents. Further variability is the one-tube/two-tube reaction issue. One-tube systems, in which the reverse transcription reaction and amplification takes place in a single tube, are clearly more convenient, however, that convenience is gained at the expense of flexibility and some sensitivity. Reagent mixes that work for both the reverse transcription step and the PCR step, are generally a compromise not optimized for either step. In this example we have chosen to run two separate reactions. The choice of the reverse transcription system is crucial to the Real-Time quantitation of specific mRNA, as the cDNA product must accurately reflect the input mRNA. Choosing a system with the sensitivity to amplify a rare mRNA, and also has the dynamic range to show differences in content, is therefore critical

The mechanism of priming is one of the most important considerations in setting up the cDNA synthesis. Oligo dT anneals to the poly A tail of mRNA to prime the reverse transcription of mRNA. This would result in some full-length transcripts and preferentially transcribe from mRNA. One obvious problem with this mechanism is that it would miss the transcripts that do not have poly A tails. There is also a tendency for incomplete reverse transcription, which results in fragments that have a higher proportion of the three prime end of transcripts and lower representations of the five prime ends. Random oligonucleotides will, by their nature, produce smaller fragments of cDNA and there may be an over-representation of the five prime ends. It may then be useful to use a mixture of oligo dT and random oligonucleotides to produce templates for real time PCR. While some of the transcripts will be incomplete, the products analyzed by PCR are relatively small (less than 200 bp) and will be well represented.

While every molecular supply company has a brand of reverse transcriptase, there are only two types of reverse transcriptase used in most assays. These are MMLV (Moloney murine leukemia virus) and AMV (avian myeloblastosis virus). Both enzymes contain RNAse H in the native form, but MMLV reverse transcriptase has much less of this RNAse. Mutated forms of MMLV are available as recombinant molecules that have reduced or absent RNAse H activity. MMLV represents the more commonly

utilized reverse transcriptase. The presence of RNAse H, reduces the amount of full-length product produced. However, the effect of RNAse H on the final sensitivity of reverse transcription and PCR analysis of transcripts is unclear. There are clearly manufacturers that claim it is harmful, and others that can show that it increases the sensitivity of the reaction. We have chosen to use the iScript reverse transcription system for our reactions, which is an RNAse H+, MMLV derived enzyme. As kit contents may vary, a detailed procedure for the reverse transcription step will not be presented.

In the current example, we utilized 2 μg of RNA from either the right or left ventricle, and reverse-transcribed, using the iScript cDNA synthesis kit (Biorad). A positive control containing RNA provided with the kit, and a negative control containing no reverse transcriptase, were cycled along with the test samples. The success of the reverse transcription reaction was assessed by PCR using the Kir 2.1 specific primers, and primers provided with the reverse transcription kit for the positive control.

Primer and Probe Design

Primer and probe design are complicated processes that require multiple parameters to be considered. The size of the product and the optimal design of both probes and primers are just starting points. The most important parameter concerning primer and probe design is the Tm gap that should exist between primer melting and probe melting. The Tm value of the primers should be between them 58–60°, whilst that of the probe should be 7–10° above that of the primers. The optimal GC contents and the ideal lengths of the primers and probes, the most favorable length of the amplicons, and the spacing that should exist between the end of the primers and the probe on the sequence to be amplified, can complicate the design process. Several commercially available software suites are available, but online sites are also available and free to use. Two such sites, which we have used, are available from Stratagene and International DNA Technologies:

- *http://labtools.stratagene.com/*
- *http://biotools.idtdna.com/gateway/*

Both programs are easy and simple to use. One simply has to cut and paste the template sequence and designate the sites one wishes the probe and primers to anneal to. The Stratagene suite enables the researcher to also design probes and primers for use in multiplexing reactions. The IDT suite does not provide this convenience, but it does allow the researcher to directly blast the chosen probe primer set to ensure their specificity for the sequence of interest, independent of the software utilized. Fortunately both software suites have their default parameters set to the ideal values for the concerns listed above, which greatly simplifies the design process even for a novice.

The Kir 2.1 primer and probe pair was designed using free web-based software provided by Stratagene. To ensure their specificity, we performed a blast search against the relevant databases. We chose to label the Kir 2.1 probe with FAM at the 5' end, and the Darkhole quencher BHQ-1 at the 3' end. Our choice of fluorophore was

for practical reasons. Firstly, the Biorad I-cycler we were using had the appropriate filters to monitor FAM and TET wavelengths. Secondly, since we anticipated multiplexing with a TET labeled cyclophilin internal control, we wanted to choose a fluorophore for the Kir 2.1 probe that would have no significant spectral overlap with TET.

Amplification

There are many enzymes available for the amplification step. These are mostly based on Taq (Thermus aquaticus) polymerase, however, others are based on mutated forms of Taq, vent polymerase (Thermococcus litoralis) and Pfu (Pyrococcus furiosus) polymerase. Taq has the highest processivity, but vent and Pfu have better fidelity, due to the presence of 3' to 5' proof reading activity. Some mutants of Taq have higher fidelity. One way to improve specificity in PCR is to utilize a hot start procedure. Most reactions are assembled at room temperature where primers may bind to DNA sequences with little specificity. As the reaction is heated to denaturation temperature, the polymerase becomes active, extending these mismatched products. Taq polymerase may be as much as 70% active at 50 °C. Mismatches can be eliminated, by preventing polymerase activation at lower temperatures (Chou et al. 1992). Many early implementations of this technique were cumbersome with wax pellets surrounding the enzyme, or the necessity of adding the polymerase after denaturation, when the reaction was already in the cycler. New implementations of hot start utilize inhibitors that are denatured at higher temperatures. Monoclonal antibodies that inactivate the enzyme are the most common mechanism (Kellogg et al. 1994). These antibodies inactivate at temperatures above 70 °C, allowing the enzyme to activate. Other implementations involve chemical inhibitors that no longer inhibit at higher temperature. However, hot start enzymes are preferable in any real time application.

In our example, PCR reactions were run using the Brilliant Core Quantitative PCR reagent (Stratagene). This cloned Taq polymerase is chemically inactivated at lower temperatures. This polymerase, as many others, comes as a "master mix", with all reagents except template, primer and probe. These mixes reduce the variability in the assay. It is useful to prepare a more complete mix with your specific probe and primers, helping to further reduce pipetting error and tube-to-tube variability.

Optimization of Probes and Primers

The molecular beacon concentration was optimized by using a standard primer concentration of 200 μM per primer and varying the final concentration of beacon from 200–500 nM.

The optimal concentration of primers for use with the molecular beacon are determined by finding the lowest concentration of forward and reverse primers which yield the lowest Ct values and adequate fluorescence. The primer concentrations are determined by holding the beacon concentration, and concentrations of either the forward or reverse constant at 200 μM, while varying the other from 200–600 nM. Af-

ter finding the lowest concentration for one of the primers, the optimal concentration for the other is found varying its concentration against the low concentration found for the first.

Multiplexing

If a researcher decides to multiplex, or amplify more than one target DNA in the same tube, the task of optimizing the Real-Time PCR reaction becomes more complex. The goal is to balance the efficiencies of the competing reactions and prevent one from completely inhibiting the other(s). For our purposes, we multiplexed the Kir 2.1 reaction and the internal control cyclophilin reaction, to minimize variability from amplifying the target and normalizing reaction in separate tubes. The Kir 2.1 and cyclophilin reactions were optimized separately, before running both reactions in the same tube. In our hands, the cylcophilin cDNA present in the right ventricle and left ventricle samples was significantly greater than that for the Kir 2.1 channel. So much so, that the cyclophilin reaction reached its exponential phase well ahead of the Kir 2.1 reaction, and thus completely inhibited the amplification of Kir 2.1. To solve this problem we created a serial dilution of the optimized cylcophilin primer concentration. We then added a master mix containing sample cDNA to the primer solution, the optimized Kir 2.1 primers and probe, as well as the optimized concentration of cyclophilin probe. By monitoring the amplification of both targets on the FAM and TET channels, we were able to determine a dilution of cyclophilin primers that could efficiently amplify its target, without preventing the concurrent amplification of Kir 2.1.

PCR Cycling Parameters

For control and experimental reactions the cycling parameters used were:
- 10 min denaturation at 95 °C
- 30 s at 95 °C
- 1 min at 55 °C X 40
- 30 s at 72 °C

Data Analysis

Relative quantification may in the end rely on the choice of a standard by which to compare the result. A variety of housekeeping genes have been used to standardize RNA analysis and there is no clear-cut advantage to any one of the standard RNA. rRNA can be used, but may not be the best choice. The amount of rRNA is higher than any expressed gene, and amounts of rRNA may vary from the amount of mRNA. There are further complications due to the lack of rRNA in purified mRNA preparations and the lack of priming by oligo-dT. The other common choices are also not perfect. These include β-actin, GAPDH, cyclophilin, β-2 microglobulin, HPRT1, and a

long list of others. All of these do vary in concentration from tissue to tissue, and may also vary due to treatment protocol. Perhaps the best approach is to measure several housekeeping genes and average their content (Vandesompele et al. 2002).

The results from the reactions in our lab are normalized against cyclophilin, and quantified using a standard curve generated from Kir 2.1 DNA. The software that accompanies many Real-Time PCR machines has the capacity to quantify target DNA relative to the standard curve automatically. Additionally, some software suites allow for automatic exportation of the Ct values into Excel for further statistical analysis. Details of the analysis software will vary by cycler and version.

Troubleshooting

Preventing Contamination

PCR in general and reverse transcription, and PCR in particular, are highly sensitive to contaminated DNA in the reaction. The two primary sources of DNA contamination are genomic DNA, which co-precipitated with the RNA, and products of other PCR reactions you may have run. To prevent the corruption of experiments, every precaution must be taken to minimize genomic DNA contamination and cross contamination between experiments. The problem of genomic is a particular one. A strategy is to design primers that span exon-exon junctions. The result is that genomic DNA will not be an effective template for the reaction. This not always practical and other mechanisms are required. Contamination of Genomic DNA can be minimized, by treating RNA samples with RNase-free DNase. This will remove the contamination, but requires total elimination of the DNase before the reverse transcription. Even trace amounts may reduce the sensitivity of the reaction.

Contamination of reactions with the products of previous ones has always been a problem with PCR. It becomes a greater problem when less abundant products are being assessed. In some ways, this problem is eliminated in Real-Time PCR, as the reaction can be tested without opening the reaction tube. However, good technique requires care in separate PCR preparation. The use of separate areas, reagents and pipettes for template preparation and amplification setup are important. Even more important, is to isolate any product from the setup reagents. The use of aerosol resistant pipette tips provides further protection, so that the product does not contain dTTP, the AmpErase system uses dUTP in the PCR reaction. UNG (Uracil-N-glycosylase) is then used, following the reverse transcription, which produces cDNA with dTTP. UNG recognizes and catalyses the destruction of DNA strands containing deoxyuridine, but not the DNA containing thymidine, preventing carryover. Thus, any DNA produced in previous amplifications can be destroyed, while the template produced for this reaction will remain intact. UNG is inactive at the temperature of a PCR reaction (greater than 50 °C).

One way to assess the level of DNA contamination in the RNA samples, is to perform a PCR reaction using total RNA that has not been reverse transcribed as a template. It is important to note that the critical parameter is not the absence of an

amplification product, but that the level of product amplified from contamination is significantly less than the reverse transcribed signal. This may be achieved with careful preparation of samples and isolation of PCR products from starting reagents.

General Considerations

As with any new technique, there are critical parameters one should be aware of to ensure success. Specificity is of paramount concern, as the amplifying of incorrect product completely nullifies PCR's utility. Since Real-Time PCR is effectively PCR with a fluorogenic probe added, all factors that could affect "non real-time amplifications" are in play. Magnesium chloride concentration and buffer composition are both factors that must be considered. Particular consideration must be made with primer composition, specificity of primer action, probe structure and melting point. Many of these parameters are controlled by the mix preparation purchased. The amounts of primers and probes as well as cycling parameters are also critical and may vary when multiplexing. In the end, the most important factors are the skill, care and consistency of the individual setting up the reactions.

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