Chapter 4

Quantitative Real-Time PCR

J. P. G. Sluijter, G. Pasterkamp, and D. P. V. de Kleijn

Changes in mRNA expression levels occur during physiological and pathological processes in the cardiovascular system. An increase in DNA transcription results in increased mRNA levels and will subsequently result in increased protein levels that regulate processes inside and outside the cell. To determine alterations in mRNA levels, traditional methods such as Northern blot and ribonuclease protection assay can be used; however, large amounts of RNA are necessary and the methods are very labor intensive. In this chapter, we focus on the newest advancements in reverse transcription polymerase chain reaction (RT-PCR) technology, the real-time PCR or quantitative PCR, using small amounts of RNA to determine expression levels. We discuss the technique in general and describe two different approaches.

1. INTRODUCTION

During physiological and pathological processes expression levels of mRNA and proteins are changed to achieve biological effects. For this, studying mRNA levels will help us to understand the underlying processes, since alteration of mRNA levels often precedes a change in protein levels, which is necessary to obtain the desired effect. Traditional methods to quantify mRNA expression levels are Northern blotting, *in situ* hybridization, ribonuclease protection, cDNA arrays, and reverse transcription polymerase chain reaction (RT-PCR). Northern analysis

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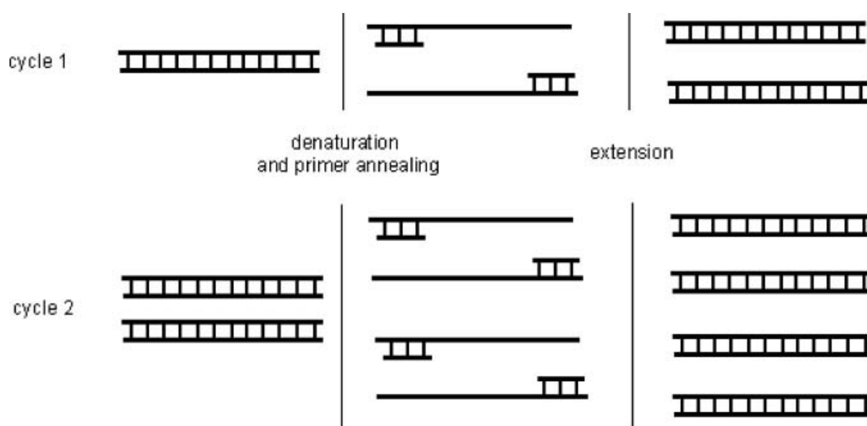

FIGURE 1. Schematic picture of DNA amplification during the PCR. Each cycle starts with a double-stranded template and denaturation is needed to create two single-strand molecules to which the primer can anneal. This serves as the starting point of the DNA polymerase and extension can occur when the right conditions are present, resulting in a doubling of the DNA template. The cycle of denaturation–annealing–extension is repeated and amplification of the template will occur.

provides information about mRNA size, alternative splicing, and the integrity of the RNA samples. The *in situ* hybridization is the only one that allows localization of the produced mRNAs (Parker and Barnes, 1999). Ribonuclease protection (RNAse protection assay) is most useful for mapping transcript initiation and termination sites (Hod, 1992). The cDNA arrays are still limited in its use by cost considerations (Bucher, 1999). The main limitation of these methods is the low sensitivity. RT-PCR is the most sensitive and accurate of the quantification methods, but needs intensive and laborious post-PCR manipulations, makes use of hazardous chemicals, and carries a potential risk for laboratory contamination.

Here we focus on one of the newest advancements in PCR technology: the real-time PCR or quantitative PCR. The introduction of this new procedure is based on fluorescence-kinetic RT-PCR and allows quantification of the PCR product in "real time." It measures PCR product accumulation during the exponential phase of the reaction. The technique is much faster than the previous endpoint RT-PCR as it is designed to provide information as rapidly as the amplification process itself, thus requiring no post-PCR manipulations.

The basic principle of the real-time PCR is the classical PCR. In short, the PCR, developed by Dr Mullis (Mullis *et al.*, 1986; Mullis and Faloona, 1987), is a rapid *in vitro* enzymatic amplification of a specific DNA region (see Fig. 1). The formation of complementary strands is the basic principle of PCR. First, the double-stranded template is denaturated to form single-stranded molecules (denaturation). Next, synthetic oligonucleotide primers, which flank the target DNA sequence that has to be amplified, hybridize to the single-stranded template and serve as the starting point of the DNA polymerase (annealing). Finally, DNA polymerase synthesizes a complementary single-stranded DNA matching the template in a process called extension.

Repeated denaturation–annealing–extension cycles will accumulate the target sequence exponentially. The reaction will continue as long as there is an excess of primers, oligonucleotides, and an active polymerase and will occur only in the 3′ direction. During the PCR, DNA amplification is exponential, eventually reaching a plateau. At any point during the exponential amplification phase for a reaction the amount of DNA can be calculated. The key is to perform this calculation before the reaction leaves the exponential phase. Modifications of the classical PCR are needed to use this technique for quantifying mRNA levels. RNA cannot serve as a template for PCR, and therefore, an essential step in determining mRNA levels is the conversion of RNA to a complementary DNA template (cDNA) by the enzyme reverse transcriptase. This cDNA is now used as the template in the PCR. The classical PCR needs analysis afterwards, which is time-consuming and not quantitative, and contaminations can occur.

Real-time PCR makes it possible to quantify the amount of target mRNA at the beginning of the PCR. This technique is very accurate, reproducible, and sensitive, with a high throughput and in which contaminations are reduced to a minimum. A well-designed real-time PCR assay can allow for the quantification of initial DNA concentration over a dynamic range of six or more orders. In real-time PCRs, fluorescent molecules are used to monitor the reaction while amplification is taking place. It is characterized by the point during amplification when the accumulation of PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles.

Real-time PCR is a very powerful technique and therefore a wide expanding technology with a range of possibilities in instrumentation and chemistries. Different competing PCR systems are developed with their own characteristics and sensitivities, e.g. the ABI Prism 7700 (Perkin-Elmer-Applied Biosytems), the Lightcycler (Roche Molecular Biochemicals), the i-Cycler iQ (Bio-Rad Laboratories) (Bustin, 2000; Giulietti *et al.*, 2001).

For detection of the amplified product, nonspecific reporter molecules (e.g. SYBR® green, ethidium bromide) as well as specific probes (e.g. molecular beacons, TaqMan™, dual-oligo FRET pairs, Scorpions™/Amplifluor™) can be used:

1. The use of nonspecific molecules will be fast and cheap and can be used for every primer set, but controls are needed to demonstrate specificity of the monitored fluorescent signal.
2. A more sensitive and specific way to monitor the amplificated signal is the use of specific hybridizing probes; however, to set up this assay is more time-consuming and more expensive.

2. NONSPECIFIC FLUORESCENT QUANTIFICATION

The use of nonspecific fluorescent molecules is a relatively simple technique and can be used for any PCR. It is based on the binding of intercalating dyes,

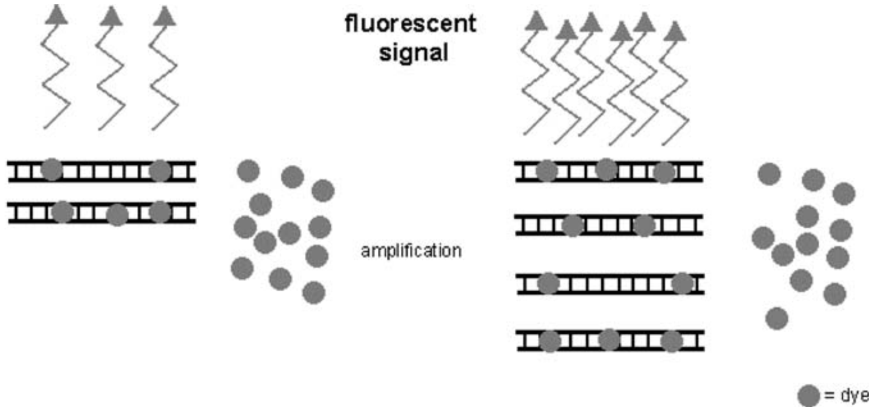

FIGURE 2. Nonspecific intercalating dye binding during amplification. Dyes bind to double-stranded DNA and when bound they will give a fluorescent emission signal. The free dye will give no signal when excited. During amplification more and more double-stranded product is formed and increasing amount of dye can bind, increasing the observed fluorescent signal.

e.g. SYBR® green and ethidium bromide, that can insert between two adjacent base pairs in a molecule of double-stranded DNA, distorting the architecture of the double helix (Morrison *et al.*, 1998). Only dye bound to DNA will give a fluorescent emission signal, and not the free dyes that are in the mixture (see Fig. 2). SYBR® green fluoresces 50 times brighter than ethidium bromide when bound to double-stranded DNA. During the PCR cycles more and more double-stranded DNA is formed; thus the amount of the dye that can bind will increase, resulting in an increased fluorescent signal. The increase in fluorescent signal will be caused by all amplified DNA products formed in your reactions, which are not necessarily specific for the desired product. When using this approach, primers must be selected carefully and PCRs must be optimized to avoid formation of nondesired products. Post-PCR correction is sometimes necessary to obtain accurate quantitative information. With this kind of DNA amplification you can measure relatively quickly and easily the expression of several genes, without the use of specific probes.

Smeets *et al.* (2003) used the nonspecific molecule SYBR® green in real-time PCR to quantify haptoglobin mRNA expression levels after balloon dilation in rabbits. Haptoglobin is an acute phase glycoprotein, mainly produced in the liver and secreted in the serum. But increasing evidence shows that haptoglobin is produced in extrahepatic tissues, e.g. the arterial wall. Haptoglobin is thought to play a role in cell migration and arterial restructuring. In this study they found an early increase in haptoglobin mRNA levels, which could be measured in the picogram range. Furthermore, they found that arterial haptoglobin consists of a unique set of glycoforms, with other oligosaccharides attached, compared to haptoglobin produced in the liver. Alterations in glycosylation are known to modify, e.g., function; thus, it is conceivable that arterial haptoglobin has a different function compared to liver-produced haptoglobin.

3. SPECIFIC FLUORESCENT QUANTIFICATION

To ensure that the increase in fluorescent signal is more sensitive and specific, you can use fluorescent oligonucleotide hybridization probes. Setting up this kind of analysis will cost some more time, but for very large studies it will be worth the investment.

One strategy often used is the TaqMan® assay (see Fig. 3A) and needs the annealing of three oligonucleotides to the DNA: two template specific primers, and a third probe that hybridizes to the target sequence (Gut *et al.*, 1999). In this

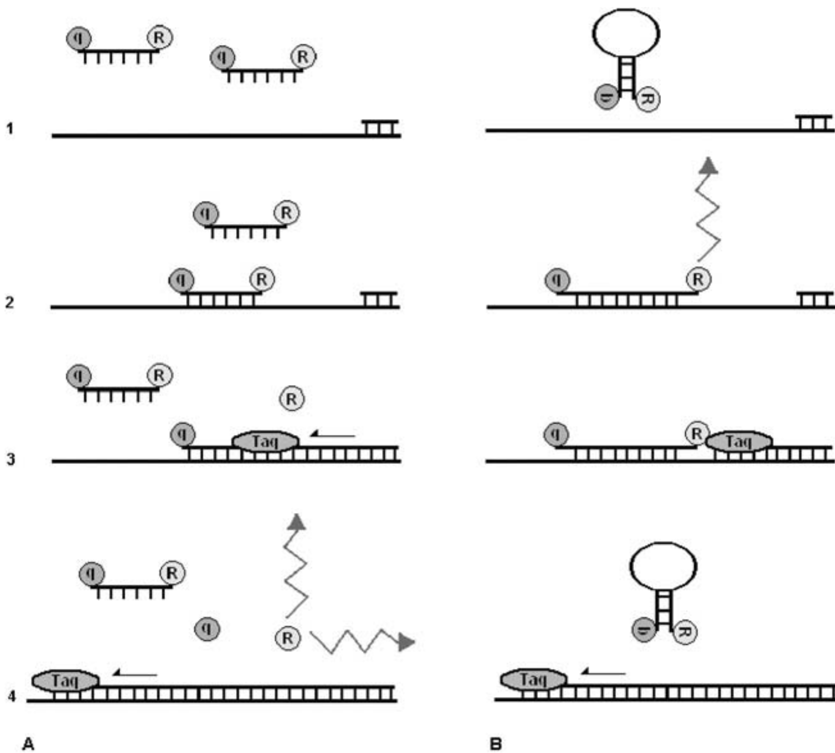

FIGURE 3. Schematic picture of the TaqMan® assay and the molecular beacon approach. (A) In the TaqMan® assay, the probe is labeled at the 5′ end with a fluorescent reporter (R) and at the 3′ end with a quencher molecule (Q). The fluorescence of the reporter will be absorbed by the quencher when the probe is still intact. During the extension phase the Taq polymerase cleaves the hybridized probe and the reporter molecule is released from the quencher. The fluorescent signal can now be detected and will be increased during amplification when more and more probe will be bound and will be cleaved. (B) The molecular beacon is a hairpin-loop-shaped oligonucleotide with a fluorescent reporter (R) to one arm and a quencher molecule to the complementary other arm. When the probe binds to the template the reporter and quencher are separated and the fluorescent signal can be monitored. During amplification more and more probe will bind and an increased fluorescent signal is observed. (*See Color Plate 8.*)

assay, which uses the 5′-nuclease activity of the DNA polymerase, the probe is labeled at the 5′ end with a fluorescent reporter and at the 3′ end with a quencher molecule and is complementary to the target DNA. The quencher molecule absorbs the energy emitted by a fluorophore and emits the energy at a different wavelength, reducing the fluorescence of the fluorophore. When the probe is still intact, the reporter and quencher are near each other and the fluorescence of the 5′-reporter is absorbed by the 3′-quencher and no signal is detected. PCR amplification of your target DNA will increase the number of probes that hybridize with the complementary template. During the extension phase of a reaction, the Taq polymerase cleaves the labeled probe which is hybridized. The reporter molecule is then released from the quencher and the fluorescent signal can be detected. Accumulation of the released reporter molecules during the amplification cycles results in an increasing fluorescent signal and is correlated to the amount of the target DNA present.

Another hybridization probe is the molecular beacon (Bonnet *et al.*, 1999; Tyagi and Kramer, 1996), which is a hairpin-loop-shaped single-stranded oligonucleotide (see Fig. 3B). It consists of a probe sequence, homologous to the target sequence, but is flanked by complimentary arm sequences, which are homologous to one another but not to the target sequence. One arm contains a fluorescent reporter and the other a nonfluorescent quencher. At room temperature, the molecular beacon assumes the hairpin formation, bringing the reporter and quencher into intimate contact; the fluorescent signal will be captured by the quencher; and no fluorescence is detected from the reporter. During the annealing step of the amplification cycle, thermodynamics favors the binding of the molecular beacon to its target rather than the formation of the hairpin structure. When the probe binds to the target sequence this will result in a separation of the reporter and quencher molecule and the fluorescent signal can be monitored. Because more and more probes will bind to the DNA, the fluorescent signal will increase during amplification. The main drawback with molecular beacons is associated with the design and costs of the hybridization probe. Large background signals are produced when the beacon folds in alternate conformations that do not place the fluorophore near the quencher or when the stem is too strong that the annealing to the target sequence will be disturbed.

Ortiz-Pallardo *et al.* (2000) used real-time PCR to identify the PiZ (proteinase inhibitor) mutations and to determine hetero- and homozygeous carrier status in whole blood and from paraffin-embedded specimens. A mutation in the α_1-antitrypsin (AAT), with the structural gene locus encoding AAT called Pi, resulting in AAT deficiency is a common inherited cause of emphysema and cirrhotic liver disease. Current diagnostic techniques are time-consuming and have a limited accuracy. They compared the results of the RT-PCR with single-strand conformational polymorphism and direct DNA sequencing, which are used nowadays. They found that the results of the RT-PCR were confirmed by the other two techniques in all cases, but allowed a higher throughput level.

4. SETTING UP A QUANTITATIVE PCR

Amplification in a real-time PCR will increase the target DNA and therefore also the observed fluorescent signal. In the early cycles of amplification, the change in fluorescence of the reporter is usually undetectable, but at some point during amplification, the accumulation of product results in a measurable change in the fluorescence of the reaction mixture. The higher the starting amount of the target DNA in your sample, the sooner a significant increase in the fluorescent signal will be registered. The cycle number observed when the fluorescent signal rises above background levels will be used to quantify the amount of DNA, and is called the threshold cycle (Gibson *et al.*, 1996). This point will always occur during the exponential phase of amplification, and therefore, quantification is not affected by any reaction components becoming limited in the plateau phase. There is a linear relationship between the threshold cycle during real-time PCR and the log of the starting amount of a template. Quantification of your target will be performed by comparison with a standard curve of your target reference standard and can be either relative or absolute. Relative quantification determines the change compared to the calibrator (standard used in dilution series) as containing either more or less mRNA. The absolute quantification allows the precise determination of copy numbers per, e.g., cell or unit mass of tissue. In most cases, the lower detection limit is 30–500 copies of target mRNA.

Computerized analysis of the data is needed to quantify the starting amounts in your samples. A grid of the reaction plate is installed to let the computer know which part of the signal represents the well that is recorded by the CCD camera. The amplification plot (see Fig. 4) displays the relative fluorescence for each well at every cycle. To determine threshold cycles and the standard curve, a PCR baseline subtraction is needed. This will correct for the fluorescent background level, the noise in your samples, and is automatically calculated from the optimal baseline cycles for each well individually or can be applied manually. After this, the standard curve can be generated and the amount of DNA in the unknown samples can be calculated. From the created standard curve, a correlation coefficient and the efficiency can be calculated. The correlation coefficient tells you something about the accuracy of the experimental data compared to the expected values, and thus how well a sample fits to the created standard curve. The efficiency of the reactions is directly related to the slope of the standard curve and gives you information about the PCR itself. An efficiency of 100% represents a doubling of your template after each cycle and corresponds with the theoretical perfect PCR. You will need equal efficiencies in the different PCRs to be able to compare different PCR runs and also normalization of your samples is needed for a relevant housekeeping gene.

5. CONCLUDING REMARKS

Using real-time PCR, one is able to monitor different expression levels of different mRNAs in one reaction (real-time multiplex gene expression) when using

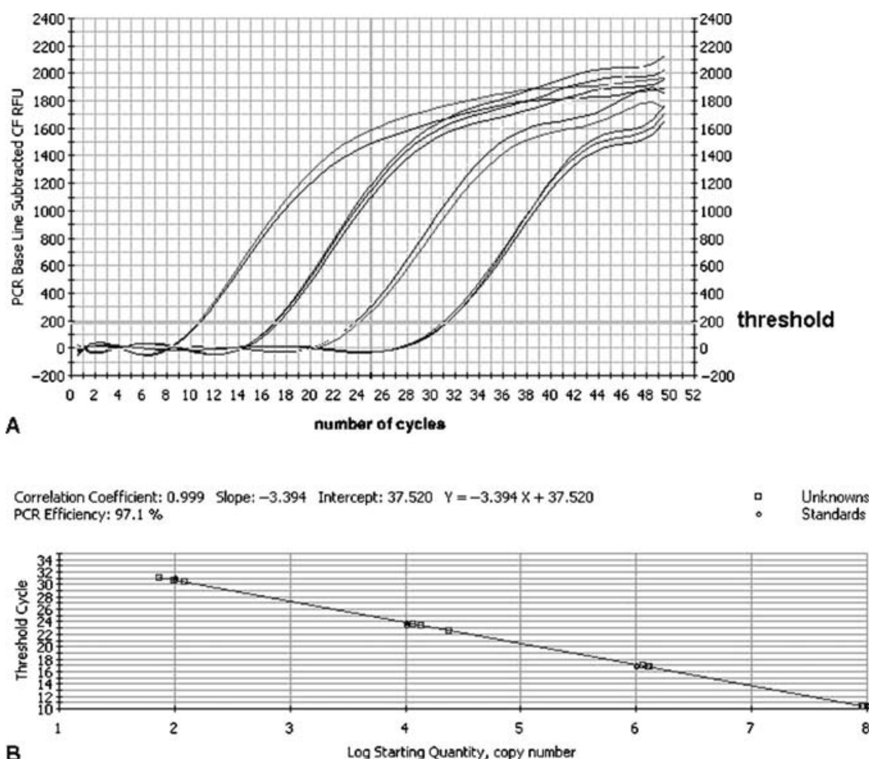

FIGURE 4. Amplification plot and standard curve generated with the iCycler iQ^{TM} software (Bio-Rad Laboratories). (A) The amplification plot displays the observed increased fluorescent signal for each well (one line represents one well). The threshold is determined by the background signal found in the first cycles and the cycle, where the observed fluorescence rises above this threshold, is called the threshold cycle and will be used in the analysis. (B) The standard curve is generated from the target reference samples and the DNA concentrations of the unknowns are calculated. The correlation coefficient is a measure of the accuracy of the data compared to the generated curve. The slope of the curve represents the PCR efficiency.

different probes with different fluorescent signals. Hybridization probes that do not cleave can identify mutations, even a single base mutation, within the PCR product. Hybridization probes can also determine the distribution of genotypes within a population compared to known genotypes and are able to detect mRNA splice variants. The real-time PCR can also be used to verify quickly the data obtained by microarray.

Quantitative, or real-time, PCR is an accurate, sensitive, and high-throughput technique that can be used for several purposes. The goal of the study determines which kind of tools is used to obtain the fluorescent data. The analysis of these data and interpretation of the results need some experience, but will be worth the investment.

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