Chapter 28
Reverse Transcription Real-Time PCR Protocol for Gene Expression Analyses

M. Taliefar, S. Bradburn, G. Podda, and C. Murgatroyd

28.1 Introduction

This protocol describes the detailed experimental procedure for real-time reverse transcription (RT) PCR using SYBR Green. Gene expression differences between healthy and atherosclerotic arteries are important to understand cellular processes involved in the progression and development of atherosclerotic disease. The sequelae of atherosclerosis are leading causes of morbidity and mortality. Much is known about the pathogenesis of atherosclerosis [1], and many studies of gene expression in atherosclerotic lesions have been performed, both at the level of single gene analysis (e.g. [2]) and by global gene expression profiling technologies such as DNA microarray (e.g. [3]). However, there are still relatively little reproducible changes on gene expression patterns in atherosclerosis. This may relate to the fact that the heterogeneous progression of atherosclerotic disease in the peripheral arteries is currently not well understood [1]. For example, it has been hypothesized that the uneven onset and progression of atherosclerosis may be explained by artery specific transcriptomes [4]. Indeed, artery specific gene expression profiles, possibly a result of differences in angiogenesis and vasculogenesis, have previously been identified and may influence atherosclerotic disease susceptibility [5]. In sum, the need for detailed examination of gene expression patterns in atherosclerosis is crucial to aid understanding.

The procedure begins with reverse transcription of total RNA. The cDNA is then used as template for real-time PCR with gene specific primers. Optimization will be needed dependent on reagents or instruments for real-time PCR.

M. Taliefar • S. Bradburn • G. Podda • C. Murgatroyd (✉)
School of Healthcare Science, Manchester Metropolitan University, Manchester, UK
e-mail: c.murgatroyd@mmu.ac.uk

M. Slevin, G. McDowell (eds.), *Handbook of Vascular Biology Techniques*,
DOI 10.1007/978-94-017-9716-0_28

28.1.1 Time Required

Time required for 20 samples:

DNA and RNA extraction:	3 h
cDNA synthesis:	3 h
Real-time PCR:	2 h
Data analysis:	1 h

28.2 Materials

- Oligonucleotide Primers *(Life Technologies™)*
- RNA
- SensiFAST™ SYBR Hi-ROX Kit *(Bioline Reagents)*
- Tetro cDNA Synthesis Kit *(Bioline Reagents)*
- RNaseZap® RNase Decontamination *(Ambion®)*
- DEPC-treated water *(Ambion®)*
- DNA LoBind 1.5 mL Tubes, PCR clean *(Eppendorf)*
- ART® self-sealing barrier pipette tips *(Sigma-Aldrich)*
- Eppendorf Mastercycler® PCR Cycler *(Eppendorf)*
- StepOnePlus™ Real-Time PCR *(Applied Biosystems®)*
- MicroAmp® Fast Optical 96-Well Reaction Plate *(Applied Biosystems®)*
- MicroAmp® 96 Well Optical Adhesive Film *(Applied Biosystems®)*
- 2100 bioanalyzer *(Agilent Technologies)*
- Gel Doc™ XR+ *(Bio-Rad Laboratories)*
- Multipurpose Centrifuge

28.2.1 Detailed Procedure

28.2.1.1 Analysis of RNA Quality and Quantity

Experiments using low-quality total RNA lead to poor results. Agarose gel electrophoresis is the conventional method for RNA analysis, but it is unreliable, labor intensive and slow. Several methods exist for quantifying RNA (Table 28.1). For RNA sample quality, the Agilent 2100 Bioanalyzer system provides sizing, quantitation and quality control of total RNA or mRNA on a single platform. This provides high quality digital data such as RNA Integrity Number (RIN) scale from 1 to 10 derived from Agilent 2100 expert software (Fig. 28.1) providing electropherogram, gel like images, histograms, dot plots and result table. RIN values range from 10 (intact) to 1 (totally degraded).

Table 28.1 Comparison of analytical and physical specification of various RNA quantification techniques

	NanoDrop 2000c	Qubit fluorometer	Bioanalyser 2100
Method	UV absorbance	Fluorescence-based dyes that bind specifically to nucleic acids	Microfluidics-based platform
Precision	3 % (0.74 at 350 nm)		10 % CV
Qualitative	NA		Nano 5–500 ng/μL; Pico 50–5,000 pg/μL; mRNA kit 25–250 ng/μL
Quantitative	0.4–15,000 ng/μL	>10 pg/μL	25–500 ng/μL
Sample amount required	2 μl	1 μl	1 μl
Number of samples per run	1	1	12 (+1 RNA ladder)
Total run time	<5 s	<5 s	30 min

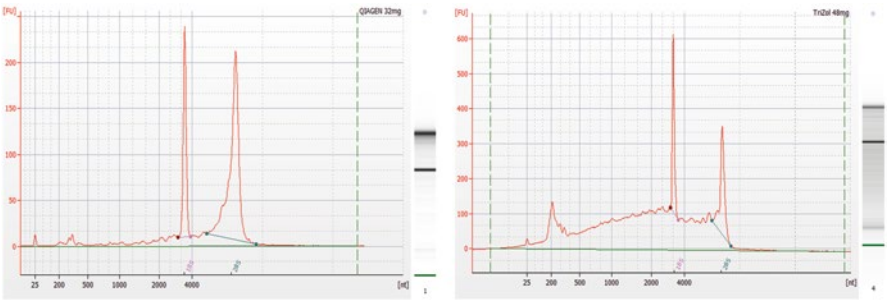

Fig. 28.1 Examples of bioanalysis electropherograms. Agilent 2100 expert software plots fluorescence intensity versus migration time and produces an electropherogram for each sample. This can also be displayed as a densitometry plot, creating a gel-like image. Intact mammalian total RNA shows two bands or peaks representing the 18S and 28S rRNA species. In general, the 28S rRNA is twice as bright and 18S rRNA. *Left Panel*; an electropherogram of a higher quality RNA preparations with a RIN value of 9.5. *Right panel*; an electropherogram of a lower quality RNA preparations with a RIN value of 5.4

28.2.1.2 Reverse Transcription

The reverse transcriptase (RT) is as critical to the success of qRT-PCR as the DNA polymerase. It is important to choose an RT that not only provides high yields of full-length cDNA, but also has good activity at high temperatures. High-temperature performance is also very important for denaturation of RNA with secondary structure. In one-step qRT-PCR, an RT that retains its activity at higher temperatures allows you to use a gene specific primer (GSP) with a high melting temperature (T*m*),

increasing specificity and reducing background. Most reverse transcriptases are derived from avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (M-MLV). Native AMV reverse transcriptase is generally more thermostable than M-MLV, but produces lower yields. However, manipulations of these native enzymes have resulted in variants with ideal properties for qRT-PCR.

When performing a two-step qPCR reaction, total or mRNA must first be transcribed into cDNA, reverse transcription is carried out with the Tetro cDNA Synthesis Kit *(Bioline Reagents)* using total RNA amounts (100 pg–2 μg). It is possible to use random hexamers or oligo dT (see Appendix 1 for comparison)

1. Vortex solutions and centrifuge briefly before use.
2. Prepare the priming premix on ice in an RNase-free reaction tube, prepare a master mix that contains all the reaction components except sample. The use of a master mix reduces the number of pipetting steps and, consequently, reduces the chances of cross-well contamination and other pipetting errors (Table 28.2).
3. Mix gently by pipetting.
4. Incubate the samples at 45 °C for 30 min. If using random hexamers, incubate at 10 min at 25 °C followed by 45 °C for 30 min.
5. Terminate reaction by incubating at 85 °C for 5 min, chill on ice.
6. If needed, store the reaction at −20 °C for long term storage, or proceed to PCR immediately (see Appendix 4 for troubleshooting)

28.2.1.3 Real-Time PCR

Before making the plate, make a layout plan for each of the samples and target genes, plus no-template control (NTC) for each target gene, DEPC traded water as a substitute for cDNA (see Appendix 3 for Control).

Normalize the primer concentrations and mix gene-specific forward and reverse primer pair. Each primer (forward or reverse) concentration in the mixture is 5 pmol/ μl.

Table 28.2 Suggested RT reaction mix composition based on 20 μl final reaction mix

	1 reaction	Master mix (20 reactions with 10 %)
Total RNA or mRNA	10 μl	NA
Primer: Oligo dT or Random Hexamers	1 μl	22 μl
10 mM dNTP mix	1 μl	22 μl
5X RT buffer	4 μl	88 μl
Ribosafe RNase inhibitor (10 u/μl)	1 μl	22 μl
Tetro reverse transcriptase (200 u/μl)	1 μl	22 μl
DEPC H_2O	2 μl	44 μl
Total volume	20 μl	220 μl

Table 28.3 Suggested PCR reaction mix composition based on 20 µl final reaction mix

	1 reaction	Master mix (20 reactions with 10 %)
SYBR green mix (2x)	10 µl	NA
Forward primer (10 µM)	1 µl	22 µl
Reverse primer (10 µM)	1 µl	22 µl
Template	4 µl	88 µl
DEPC H_2O	2 µl	44 µl
Total volume	20 µl	220 µl

Table 28.4 Suggested real-time PCR condition for SensiFAST SYBR HI-Rox kit (Bioline)

Cycles	Temp	Time	Data collection	Notes
1	95 °C	2 min	OFF	Polymerase activation
40	95 °C	5 s	OFF	Denaturation[a]
	60–65 °C	10 s	ON	Annealing[b]
	72–72 °C	5–20 s	OFF	Extension[c]
Melt Curve	95 °C	15 s	OFF	
	60 °C	1 min	OFF	
	60 °C + 3 °C		ON	
	95 °C	15 s	ON	

[a]Denaturation: high temperature incubation is used to melt double-stranded DNA into single strands and loosen secondary structure in single-stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 95 °C). The denaturation time can be increased if template GC content is high

[b]Annealing: during annealing, complementary sequences have an opportunity to hybridize, so an appropriate temperature is used that is based on the calculated melting temperature (T*m*) of the primers (5 °C below the T*m* of the primer)

[c]Extension: At 70–72 °C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in real-time PCR is small, this step is often combined with the annealing step using 60 °C as the temperature

Prepare the following mixture (for 20 µl) in each optical tube (Table 28.3).

Start PCR cycle:

Depending on primers condition the cycling stage were set, the SYBR manufactures suggested real-time PCR condition were followed (Table 28.4).

After PCR is finished, remove the tubes from the machine. Examine the melting curves and amplification plots. Export the data from StepOne™ software to digital spreadsheet and analyze the real-time PCRs (*see "Quantitation of Results"*).

A melting curve of each reaction allows one to check the specificity of the reaction (Fig. 28.2). The melting curve charts the change in fluorescence observed when double-stranded DNA with incorporated dye molecules (i.e., SYBR green) dissociates (i.e. melts) into single-stranded DNA as the temperature of the reaction is raised. This is seen as a sudden decrease in fluorescence detected when the melting point (T*m*) is reached, due to dissociation of the DNA strands and subsequent release of the dye. Melting curves allow one to detect multiple peaks or an abnormal amplification plot suggesting either unspecific or inefficient PCR reactions. Primer-dimers can

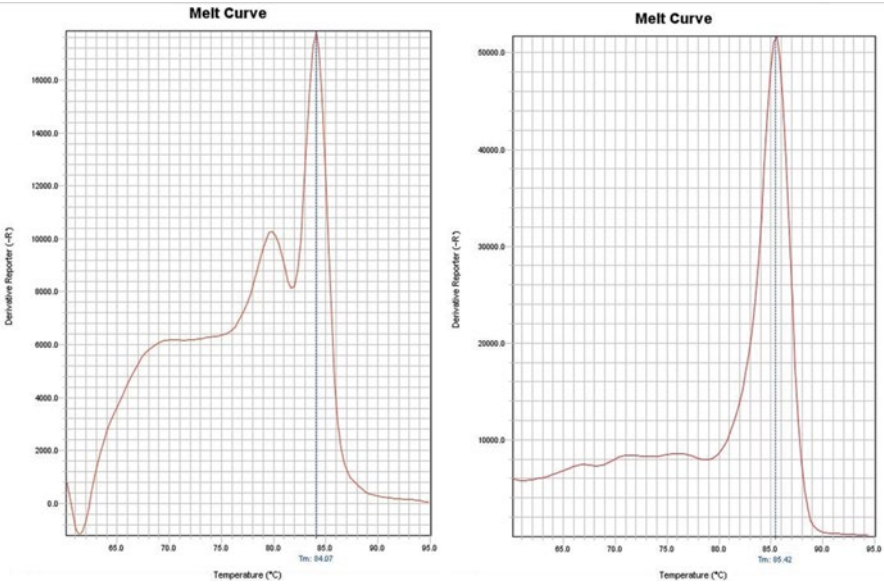

Fig. 28.2 Examples of melting curves resulting from real-time PCR reactions. *Left panel;* A melt curve plot representing unspecific primer binding resulting in multiple product formation. *Right panel;* An ideal melt curve produces one sharp peak indicating suitable primer specificity of the desired target

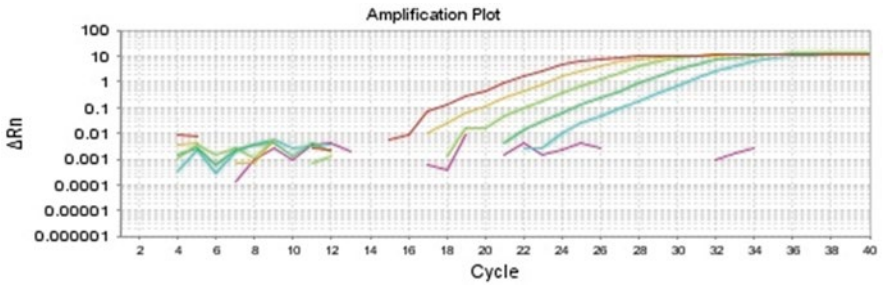

Fig. 28.3 A typical amplification plot from a set of real-time PCR reactions. Amplification plots are created when the fluorescent signal from each sample is plotted against cycle number. This can be used to extrapolate the Ct (cycle threshold) value. The incomplete plots at the start of the reaction represent background signals

also be detected as shown by additional peaks to the left of the peak for the amplified product in the melt curve. The PCR specificity can be further examined by running 5 μl of the PCR product on a 2.5 % agarose gel with a suitable DNA ladder.

Amplification plots are created when the fluorescent signal from each sample is plotted against cycle number. Therefore, amplification plots represent the accumulation of product over the duration of the real-time PCR experiment (Fig. 28.3). During the early cycles of the PCR reaction, there is little change in the fluorescent signal.

As the reaction progresses, with each cycle fluorescence begins to increase. A threshold is set and used to assign the threshold cycle, or Ct value, of each amplification reaction. Ct values are inversely related to the amount of starting template: the higher the amount of starting template in a reaction, the lower the Ct value for that reaction.

28.3 Quantitation of Results

Two strategies are commonly employed to quantify the results obtained by real-time RT-PCR; the standard curve method and the comparative threshold method.

The Standard Curve Method involves constructing a standard curve from an RNA of known concentration. This curve is then used as a reference standard for extrapolating quantitative information for mRNA targets of unknown concentrations. Though RNA standards can be used, their stability can be a source of variability in the final analyses. In addition, using RNA standards would involve the construction of cDNA plasmids that have to be in vitro transcribed into the RNA standards and accurately quantitated, a time-consuming process.

The Comparative Ct Method involves comparing the Ct values of the samples of interest with a control or calibrator such as a non-treated sample or cDNA from normal tissue. The Ct values of both the calibrator and the samples of interest are normalized to an appropriate housekeeping gene (see Appendix 2). The comparative Ct method is also known as the $2^{-\Delta\Delta CT}$ method:

$$\Delta\Delta C_T = \left(C_{T\,\text{Sample}} - C_{T\,\text{Sample housekeeping}}\right) - \left(C_{T\,\text{Reference}} - C_{T\,\text{Reference housekeeping}}\right)$$

ΔC_T sample is the Ct value for any sample normalized to the housekeeping gene and ΔC_T reference is the Ct value for the calibrator also normalized to the housekeeping gene. To obtain tight data from RT-PCR experiment should ideally be prepared in replicates (triplicates), $2^{-\Delta\Delta CT}$ results used to perform independent *t*-test using IBM-SPSS or Microsoft Excel software.

Appendices

Appendix 1

Picking Random Primers and Oligo dT

Oligo dT primers are a favorite choice for two-step cDNA synthesis reactions because of their specificity for mRNA and because they allow many different targets to be studied from the same cDNA pool. However, because they always initiate reverse transcription at the 3′ end of the transcript, difficult secondary

structure may lead to incomplete cDNA synthesis. Oligo dT priming of fragmented RNA, such as that isolated from Formalin-Fixed Paraffin-Embedded (FFPE) samples, may also be problematic. Nonetheless, as long as the primers are designed near the 3′ end of the target, premature termination downstream of this location is less of an issue.

Random primers are useful for synthesizing large pools of cDNA. They are also ideal for non-polyadenylated RNA, such as bacterial RNA, because they anneal throughout the target molecule. Degraded transcripts such as FFPE samples and secondary structure within the RNA do not pose as big a problem with random primers as they do with gene-specific primers and oligo dT primers. While increased cDNA yield is a benefit, data has shown that random primers can overestimate copy number when used in real-time RT-PCR experiments. Employing a combination of random and oligo dT primers can sometimes increase data quality by combining the benefits of both if used in the same first-strand cDNA synthesis reaction. Random primers are used only in two-step qRT-PCR reactions.

Appendix 2

Picking Housekeeping Gene

Relative quantification is a powerful technique that is commonly used to study RNA gene expression. In relative quantification the expression of a target gene is measured with respect to a stably expressed reference gene (so-called housekeeping gene); the two gene levels are expressed as a ratio.

Housekeeping genes must meet certain criteria before they can be effective reference genes. Housekeeping genes encode proteins that are essential for maintenance of cell function. For instance, housekeeping genes which code for components of the cytoskeleton (e.g., beta-actin, alpha-tubulin), components of the major histocompatibility complex (such as beta-2-microglobulin), enzymes of the glycolytic pathway (GAPDH (glyceraldehyde-3-phosphate dehydrogenase)) or ribosomal subunits appear to be expressed ubiquitously. However, several reports indicate that the expression of housekeeping genes is actively regulated; levels may vary across tissues and different types of cells, during cell proliferation and stages of development, or due to experimental treatment of cells.

Therefore, when choosing a housekeeping gene as a reference for relative quantification, one must identify a gene whose expression level remains relatively constant for a certain experimental setup. In fact, it is usually necessary to test a panel of housekeeping genes experimentally to find one that is not regulated in the investigated system. Since choosing an appropriate reference gene is critical for accurate quantitative RNA analysis, the behavior of candidate genes in different cell types and cell metabolic stages should be carefully examined.

Appendix 3

No-Template Control (NTC)

Controls in real-time PCR reactions prove that signal obtained from experimental samples represent the amplicon of interest, thereby validating specificity. All experiments should include a no-template control (NTC), and qRT-PCR reactions should also include a no-reverse transcriptase control (no-RT). NTC controls should contain all reaction components except the cDNA sample. Amplification detected in these wells is due to either primer-dimers or contamination with completed PCR reaction product. This type of contamination can make expression levels look higher than they actually are. No-RT reactions should contain all reaction components except the reverse transcriptase. If amplification products are seen in no-RT control reactions, this indicates that DNA was amplified rather than cDNA. This can also artificially inflate apparent expression levels in experimental samples

Appendix 4

Troubleshooting (Listed Some Major Causes for Real-Time PCR Failures)

Little or no PCR product: Poor quality of PCR templates, primers, or reagents may lead to PCR failures. First, please include appropriate PCR controls to eliminate these possibilities. Some genes are expressed transiently or only in certain tissues. In our experience, this is the most likely cause for negative PCR results. Please read literature for the gene expression patterns. One caveat is that microarrays are not always reliable at measuring gene expressions. After switching to the appropriate templates, we obtained positive PCR results in contrast to the otherwise negative PCRs (see our paper for more details).

Poor PCR amplification efficiency: The accuracy of real-time PCR is highly dependent on PCR efficiency. A reasonable efficiency should be at least 80 %. Poor primer quality is the leading cause for poor PCR efficiency. In this case, the PCR amplification curve usually reaches plateau early and the final fluorescence intensity is significantly lower than that of most other PCRs. This problem may be solved with re-synthesized primers.

Primer dimmer: Primer dimer may be occasionally observed if the gene expression level is very low. If this is the case, increasing the template amount may help eliminate the primer dimer formation.

Multiple bands on gel or multiple peaks in the melting curve: Agarose gel electrophoresis or melting curve analysis may not always reliably measure PCR specificity. From our experience, bimodal melting curves are sometimes observed

for long amplicons (>200 bp) even when the PCRs are specific. The observed heterogeneity in melting temperature is due to internal sequence inhomogeneity (e.g. independently melting blocks of high and low GC content) rather than non-specific amplicon. On the other hand, for short amplicons (<150 bp) very weak (and fussy) bands migrating ahead of the major specific bands are sometimes observed on agarose gel. These weak bands are super-structured or single-stranded version of the specific amplicons in equilibrium state and therefore should be considered specific. Although gel electrophoresis or melting curve analysis alone may not be 100 % reliable, the combination of both can always reveal PCR specificity in our experience.

Non-specific amplicons: Non-specific amplicons, identified by both gel electrophoresis and melting curve analysis, give misleading real-time PCR result. To avoid this problem, please make sure to perform hot-start PCR and use at least 60 °C annealing temperature. We noticed not all hot-start Taq polymerases are equally efficient at suppressing polymerase activity during sample setup. The SYBR Green PCR master mix described here always gives us satisfactory results. If the non-specific amplicon is persistent, you have to choose a different primer pair for the gene of interest.

References

1. Slevin M (2011) Therapeutic angiogenesis for vascular disease: molecular mechanisms and targeted clinical approaches for the treatment of angiogenic disease. Springer, Dordrecht. ISBN 978-90-481-9494-0
2. Liu J, Sukhova GK, Sun JS, Xu WH, Libby P, Shi GP (2004) Lysosomal cysteine proteases in atherosclerosis. Arterioscler Thromb Vasc Biol 24:1359–1366
3. Eyster KM, Appt SE, Mark-Kappeler CJ, Chalpe A, Register TC, Clarkson TB (2011) Gene expression signatures differ with extent of atherosclerosis in monkey iliac artery. Menopause 18(10):1087–1095
4. Knowles JW, Assimes TL, Li J, Quertermous T, Cooke JP (2007) Genetic susceptibility to peripheral arterial disease: a dark corner in vascular biology. Arterioscler Thromb Vasc Biol 14(10):2068–2078
5. Burridge KA, Friedman MH (2010) Environment and vascular bed origin influence differences in endothelial transcriptional profiles of coronary and iliac arteries. Am J Physiol Heart Circ Physiol 299:H837–H846