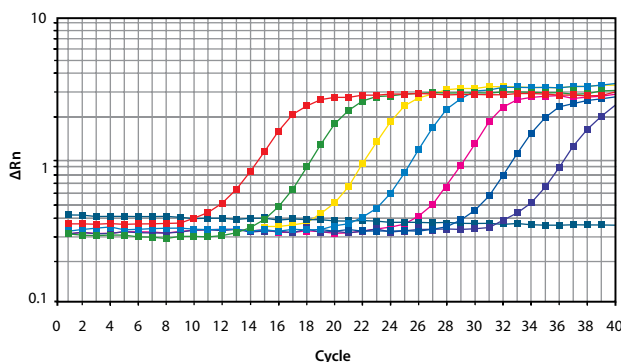


Protocol: Quantitative PCR for Determination of Angiogenic Factors

Introduction

In recent years Quantitative PCR has reached a level of sensitivity, accuracy, and ease to support use as a routine assay for measuring gene level expression. The field of cancer research is currently validating a number of applications showing that qPCR can be a reliable tool for both researchers and clinicians concerning the behavior of tumors.¹ Quantitative PCR is one of the molecular techniques providing the tools necessary to investigate tumor biology and to discover the genetic and epigenetic causes of cancer. qPCR is being used to analyze the biological differences between tumors that account for variations in morphology and clinical behavior. Quantitative PCR is playing an increasingly important role in clinical testing, providing information about gene expression, gene amplification or loss, and small alterations. It is also being used for detection and quantification of viral causes of cancer. qPCR has proven to be an extremely valuable diagnostic resource due to its objectivity, speed, versatility, and cost-effectiveness.²

Quantitative PCR uses the linearity of DNA amplification to determine absolute or relative amounts of a known sequence in a sample. By using a fluorescent reporter in the reaction, it is possible to measure DNA generation. In qPCR, DNA amplification is monitored at each cycle of PCR. When the DNA is in the log linear phase of amplification, the amount of fluorescence increases above the background. The point at which the fluorescence becomes measurable is called the Threshold cycle (C_T) or crossing point. By using multiple dilutions of a known amount of standard DNA, a standard curve can be generated of log concentration against C_T . The amount of DNA or cDNA in an unknown sample can then be calculated from its C_T value.



C_T values for the lambda amplicon using SYBR[®] Green JumpStart[™] Taq ReadyMix[™]. Quantitative PCR (qPCR) was performed on pBac-2cp. Initial template copy number was 10^6 and was diluted 10-fold in subsequent wells. Threshold cycles (C_T) were determined using the ABI PRISM 7700 Sequence Detection software, and were found to be 15.304 (10^6), 18.848 (10^5), 22.883 (10^4), 26.208 (10^3), 29.821 (10^2), 33.398 (10^1), 37.038 (10^0), and 40 (0).

Methods of Quantification

Standard Curves

Standard curves are necessary for both absolute and relative quantification. When generating standard curves, different concentrations of DNA (typically five) should be used to generate a standard curve that will bracket the concentration of the unknown. Each concentration should be run in duplicate.

Absolute and Relative Quantification

Reagents and kits for quantitative PCR may be used to quantify target DNA using either absolute or relative quantification. Absolute quantification techniques are used to determine the amount of target DNA in the initial sample, while relative quantification determines the ratio between the amount of target DNA and a reference amplicon. The ideal reference amplicon would have invariant, constitutive expression. In practice, a housekeeping gene is typically chosen for this function, but there are other reference choices which better adhere to the above requirements.³

Absolute quantification uses external standards to determine the absolute amount of target nucleic acid. To remove the differences in quantification due to annealing, the primer binding sites of the external standards must be the same as those in the target sequence. The ideal external standard contains sequences that are the same as or which vary only slightly from the target sequence. Equivalent amplification efficiencies between the target and external standard are necessary for absolute quantification. Once a suitable construct or amplicon is identified, a standard curve of external standard dilutions is generated and used to determine the concentrations of unknown target samples.

Relative quantification calculates the ratio between the amount of target template and a reference template in a sample. Since this method measures the amount of target relative to a presumably invariant control, relative qPCR is most often used to measure genetic polymorphism differences, for instance, between tissues or between healthy and diseased samples. The advantage of this technique is that using an internal standard can minimize variations in sample preparation and handling.

The accuracy of relative quantification depends on the appropriate choice of a reference template for standards. Variability of the standard will influence the results and so it is most important that standards be appropriate.³ Some researchers choose not to run a standard curve and report target quantities as a fraction of the reference, a technique termed comparative quantitation. Alternatively, one may assume that the difference in amplification efficiencies of target and reference is negligible and quantify the target based solely on the standard curve determined for the reference sequence. Finally, in the most accurate of the relative quantification techniques, the amplification efficiencies of both the reference and target are measured, and a correction factor is determined. This process, termed normalization,³ requires a sample containing known concentrations of both target and reference and the generation of two standard curves.



Determination of PCR Reaction Efficiencies

The PCR efficiency between a reference sample and a target sample is determined by preparing a dilution series for each target. The C_T values from either the reference or target is then subtracted from the other. The difference in C_T values is then plotted against the log of the template amount. If the resulting slope of the straight line is less than ± 0.1 , the amplification efficiencies are similar.

References

1. Mocellin, S., et al., Quantitative real-time PCR in cancer research. *Arch. Immunol. Ther. Exp. (Warsz)*, **51**, 301-13 (2003).
2. Bernard, P.S. and Wittwer, C.T., Real-time PCR technology for cancer diagnostics. *Clin. Chem.*, **48**, 1178-85 (2002).
3. Bustin, S.A., Quantification of mRNA using real time reverse transcription PCR (RT-PCR): trends and problems, *J. Mol. Endocrinol.* **29**, 23-9 (2002).

Procedure

For best results, optimal concentrations of primers, probes, $MgCl_2$, KCl and PCR enhancers need to be determined. Testing various combinations of primer concentrations (50-1000 nM) while keeping the probe concentration constant (250 nM) is most efficient for primer optimization. The same method could be used to optimize probe concentrations by varying probe concentrations (50-250 nM) and keeping optimal primer concentrations constant. If maximum sensitivity is not required and your PCR target is abundant, satisfactory results for probe-based qPCR are often obtained with final concentrations of both primers at 500 nM and probe at 250 nM.

Procedure for Routine Analysis

1. Preparation of a reaction master mix is highly recommended to give best reproducibility. Mix all reagents but template in a common mix, using ~10% more than needed. Once template is diluted into the reaction vessel, master mix is aliquoted into the proper tube or plate for thermocycling.

Volume*	Reagent	Final Concentration
25 μ L	2X JumpStart Taq ReadyMix	1.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTP, stabilizers
(0.5 μ L)	Reference Dye** (optional)	1x
-- μ L	Forward Primer	50-1000 nM
-- μ L	Reverse Primer	50-1000 nM
-- μ L	Template DNA	10 ng-100 ng
q.s. to 50 μ L	Water	
50 μ L	Total Volume	

* Volume for 50 μ L reaction, however component volumes may be scaled to give the desired reaction volumes.

** Use 0.1x for ABI 7500 and Stratagene instruments; replace with FITC for BioRad iCycler.

2. Mix gently by vortexing and briefly centrifuge to collect all components at the bottom of the tube.
3. Perform thermal cycling.

Optimal cycling parameters vary with probe design and thermal cycler. Check your thermal cycler manual. It may be necessary to optimize the cycling parameters to achieve maximum product yield and/or quality.

Typical Cycling Parameters for 100 bp - 600 bp Fragments

This protocol has been used successfully with the following thermal cyclers: Stratagene MX 3000P*, BioRad iCycler, and ABI 7700.

Note: Dual labeled probes are usually designed for two step 94 °C/60 °C cycling conditions. Other probes are designed for a three step regimen, with an annealing temperature of 55 °C (probe T_m ~ 60 °C). Use the cycling conditions for which your probes were designed.

	Temperature	Time
Initial denaturation	94 °C	2 min.*
40 cycles:		
Denaturation	94 °C	15 sec
Annealing/Extension	60 °C or 5 °C below lowest primer T_m	1 min **
(Optional) Hold	4 °C - only if products will be run out on a gel	

* Initial denaturation of greater than two minutes is not recommended, and is unnecessary.

** Detection is usually accomplished at this step.

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Reagents for Quantitative PCR and RT-PCR

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- Standard qPCR - kits for plate-based and tube-based instruments
- Capillary tube qPCR - kits for the Roche LightCycler[®] 2.0 & 1.0
- High-Throughput qPCR - kits with reference dye premixed into the ReadyMix. Compatible with most ABI instruments
- One-Step qRT-PCR - kits that include reverse transcriptase for streamlined quantification of RNA levels

SYBR[®] Green Detection

Standard qPCR

Compatible Platforms: ABI instruments; Roche LightCycler[®] 480; Bio-Rad/MJ instruments; Stratagene instruments; Corbett Rotor-Gene 6000 and Rotor-Gene 3000; Eppendorf Mastercycler[®] ep realplex instruments

Name	Cat. No.
SYBR [®] Green JumpStart [™] Taq ReadyMix [™]	S4438-20RXN S4438-100RXN S4438-500RXN
SYBR [®] Green JumpStart [™] Taq ReadyMix [™]	S5193-20RXN S5193-100RXN S5193-400RXN

Capillary tube qPCR

Compatible Platforms: Roche LightCycler 2.0 & 1.0

Name	Cat. No.
SYBR [®] Green JumpStart [™] Taq ReadyMix [™] for Quantitative PCR, Capillary Formulation	S1816-100RXN S1816-400RXN
SYBR [®] Green JumpStart [™] Taq ReadyMix [™]	S5193-20RXN S5193-100RXN S5193-400RXN

High Throughput qPCR

Compatible Platforms: ABI 7300, 7700, 7900, and StepOne[™]

Name	Cat. No.
SYBR [®] Green JumpStart [™] Taq ReadyMix [™] for High Throughput QPCR	S9194-20RXN S9194-400RXN S9194-2000RXN

One-Step qRT-PCR

Compatible Platforms: ABI instruments; Roche LightCycler 480; Roche LightCycler 2.0 & 1.0; Bio-Rad/MJ instruments; Stratagene instruments; Corbett Rotor-Gene 6000 and Rotor-Gene 3000; Eppendorf Mastercycler ep realplex instruments

Name	Usage	Cat. No.
SYBR [®] Green Quantitative RT-PCR Kit	100 PCR reactions (50 µl)	QR0100-1KT

Probe-based Detection

Standard qPCR

Compatible Platforms: ABI instruments; Roche LightCycler 480; Bio-Rad/MJ instruments; Stratagene instruments; Corbett Rotor-Gene 6000 and Rotor-Gene 3000; Eppendorf Mastercycler ep realplex instruments

Name	Cat. No.
JumpStart [™] Taq ReadyMix [™] for Quantitative PCR	D7440-20RXN D7440-100RXN D7440-400RXN
JumpStart [™] Taq ReadyMix [™] with dUTP	D9191-20RXN D9191-100RXN

High-Throughput qPCR

Compatible Platforms: ABI 7300, 7700, 7900, and StepOne

Name	Cat. No.
JumpStart [™] Taq ReadyMix [™] for High Throughput Quantitative PCR	D6442-20RXN D6442-400RXN D6442-2000RXN

One-Step qRT-PCR

Compatible Platforms: ABI instruments; Roche LightCycler 480; Roche LightCycler 2.0 & 1.0; Bio-Rad/MJ instruments; Stratagene instruments; Corbett Rotor-Gene 6000 and Rotor-Gene 3000; Eppendorf Mastercycler ep realplex instruments

Name	Usage	Cat. No.
Quantitative RT-PCR ReadyMix [™]	100 PCR reactions (50 µl)	QR0200-1KT

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