

Current challenges of real-time PCR quantitation.

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Introduction

A plethora of protocol enhancements and analytical applications soon followed the advent of the polymerase chain reaction (PCR) in 1985ⁱ. However, analysis of amplification products subsequent to completion of the PCR process was at that time limited to electrophoretic separation and fluorescence detection. In 1992, Higuchi and colleagues developed a real-time method for the simultaneous amplification and visualization of PCR productsⁱⁱ. For the first time in seven years, the kinetics of PCR amplification could be monitored and recorded. Initially, real-time quantitative PCR (RQ-PCR) required the addition of ethidium bromide to each amplification reaction, a thermal cycler equipped with an ultraviolet light source to irradiate the intercalator dye and a computer-controlled cooled CCD camera for detection of the increasing fluorescent signal generated with each cycle of amplification. The fluorescence intensity of ethidium bromide increases as it binds double-stranded DNA and PCR generates increasing amounts of double-stranded DNA with each round of amplification. A plot illustrating the increase in fluorescence observed with each cycle of amplification provides a much more useful and comprehensive picture of the PCR process than a single data point as previously measured for the total accumulation of product after a fixed number of cycles. It quickly became apparent however, that the use of intercalators as reporters of PCR product accumulation had its drawbacks as non-specific PCR products contributed to the overall fluorescent signal collected in some reactions. Overtime, the use of probe-based PCR product detection has gained a lot more popularity than that of intercalating dyes due to its specificity and the ease of data analysis. As expected, a number of probe-based detection methods have been introduced into the market all claiming higher specificity, ease of use and design, reduced reagent cost and improved signal sensitivity for low copy amplification targets. In principle, most of these methodologies are quite similar in that output fluorescence signal is produced only as a result of hybridization between a sequence-specific probe(s) and its complementary amplicon target.ⁱⁱⁱ

Applications and Challenges

Microbiology and Virology

Real-time PCR has become a powerful tool in the area of infectious disease testing, most notably for the detection and genotype determination of various microorganisms as well as in the calculation of viral load.^{iv} Use of RQ-PCR in this field has gained much acceptance over the last few years primarily because it is a rapid, sensitive, reproducible, and easy to use technology, but most importantly, it minimizes the risk of carryover contamination because amplification and detection occur within a closed system.

Compared to competitive PCR and other conventional PCR approaches to measuring viral load, RQ-PCR has become a more attractive alternative for a number of reasons.

First, it is easier to design and optimize real-time PCR reactions than it is to reproducibly setup reactions requiring the co-amplification of an internal competitive sequence of known concentration as is the case with quantitative competitive PCR reactions. In addition, RQ-PCR offers a broader range of detection in terms of starting DNA template quantities. A 10^{7-8} fold titration series can easily be monitored by RQ-PCR and the data collected are much more accurate and extensive than those which rely on simply measuring the end-point PCR products generated at the completion of a reaction. In a clinical laboratory setting the ability to process samples with varying amounts of input nucleic acid is an anticipated prerequisite for any method under evaluation. In comparison with traditional viral culture and hybridization-based methods of detection, RQ-PCR offers a comparable, if not higher, analytical sensitivity.

Despite the many advantages that RQ-PCR has to offer in the analysis and detection of infectious agents, there are a number of challenges that the user must be aware of in order to employ the technology appropriately in the laboratory. Some of the issues have been cleverly tackled by some investigators, but others remain challenges that will need to be addressed by the technology as it continues to develop and expand in its application and scope. One major constraint of RQ-PCR is its limited capacity for multiplexing. This drawback is in part due to limitations set by the hardware and software currently available for the platform along with the complexity of design associated with existing probe-based chemistries. Most instruments use a single excitation wavelength and the commercial availability of fluorophores with non-overlapping discernable emission spectra is limited. The ability to multiplex real-time PCR reactions in a single tube would improve the cost of testing and throughput of the technology and enhance the quantitative aspect of RQ-PCR by allowing the use of multiple internal amplification markers. The nature of these markers is a topic of discussion in another section of this document. The need for multiplexing also stems from the difficulty in analyzing highly polymorphic nucleic acid sequences in which assay specificity may be compromised by the presence of multiple sequence variants at a particular locus. Another factor contributing to the limited multiplexing capacity of RQ-PCR is the inability to measure amplicon size. This feature is one that allows extensive multiplex capability to end-point gel analysis of conventional PCR products. This intrinsic limitation makes RQ-PCR a less desirable choice than conventional multiplexed PCR for example in discriminating between multiple viral genotypes within a single reaction. Although improvements in the instrumentation are slowly being introduced into the market to allow multiple simultaneous color detection (more than four dyes), the challenge of running multiple reactions in one tube will need to be addressed more skillfully as the need will be driven by economics in many laboratories. One approach currently used to maximize the discriminating power of RQ-PCR is melt-curve analysis. The basic idea behind this type of analysis is that the denaturing properties of intercalator-bound double-stranded DNA amplicons or probe-template hybrids will differ based on differences in length and sequence. Dissociation kinetics can be monitored by changes in fluorescent intensity, which typically translates to a decrease in fluorescence signal as duplexes melt.

The ability to multiplex RQ-PCR reactions will be extremely important in the fields of oncology and genetic disease testing. As microarray data generates genetic profiles for

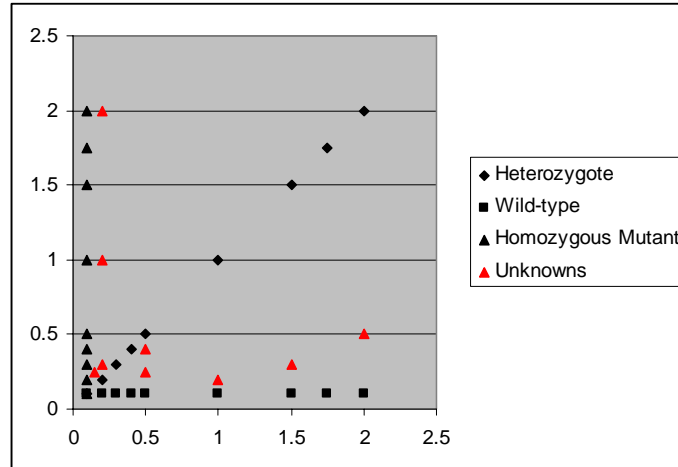
various disease states, these gene panels will require further evaluation by RQ-PCR. Due to constraints related to cost and limited DNA or RNA sample quantities, RQ-PCR assays will only be a viable option if their multiplexing capabilities are extended. Running a panel of 50-100 genes in duplicate as single reactions is not cost effective and for limited tissue samples, it is simply not a feasible option.

As a general rule, good laboratory practices must be followed when performing RQ-PCR. Slight differences in pipetting can yield vastly different quantitative results. Although automation strategies have helped to overcome this often overlooked source of error, many laboratories still experience assay variability, perhaps without being aware of it, as a result of “multiple user” syndrome. Other careful practices often disregarded include the use of optimal chemistry, thorough quality control of all starting reagents, avoidance of air bubbles (introduced by pipetting or mixing) in the reaction, centrifugation to avoid droplets from adhering to the sides of the reaction vessel, not writing on the PCR plate, thorough maintenance and calibration of instruments. All of these factors can have adverse effects on the PCR reaction either by altering the fluorescence signal or inhibiting the PCR reaction. One major source of error emanates from the amplification of extremely low copy templates. The precision of the technology decreases as a function of input nucleic acid template. While duplicates of high copy templates overlap almost perfectly in terms of their amplification kinetics, duplicates of reactions in which the target sequence is limiting (low viral load or paraffin-embedded tumor samples) demonstrate very poor reproducibility due to sampling error and mathematical binding probabilities. Quantitative measurements from low copy starting templates should be avoided when possible.

Genetic Disease

SNP Detection

Several laboratories use real-time PCR technology as a method for SNP or mutation detection. This approach is quick, convenient and relatively inexpensive as the slightly higher reagent costs are offset by the reduced labor-associated costs. One challenge that clinical diagnostic laboratories encounter is the ability to generate positive control samples to run with these assays. Although this is not a challenge intrinsic to the technology, it is complicated by the fact that real-time PCR is a highly sensitive technology platform and the use of plasmid clones or other synthetic templates can be challenging as these do not often mimic the behavior of genomic DNA samples at the same stoichiometric concentrations. In assays requiring highly specific discrimination between two alleles of similar sequence, such as a deletion along a homopolymer track, design can be quite challenging in the absence of controls for rare mutations. In addition, low input DNA quantity may introduce additional challenges in the interpretation of data. As the input DNA quantity decreases, the plot of wild-type to mutant signal becomes less discriminating between genotypes. This is depicted in the diagram below:



The confidence interval for calling point (2X , 0.5Y) a wild-type homozygous sample in the graph above is greater than that of calling the same genotype for point (0.5 X, 0.25Y). Strict input DNA requirements must be set in order to ensure that results will fall above a minimum relative fluorescent intensity of 1.0 for both wild-type and mutant probe signals, if present.

The allelic discrimination (AD) methodology can in some cases be an expensive alternative to the many other SNP detection methodologies currently available. In the clinical laboratory setting, current procedural terminology or CPT-based reimbursement for Taqman AD assays is inadequate compared to that of other technologies which employ procedures more commonly described by the molecular CPT reimbursement guidelines. In addition, AD assays using real-time PCR technology become challenging to run in a high-throughput environment in which processing large sample volumes is often coupled with the analysis of large mutation panels.

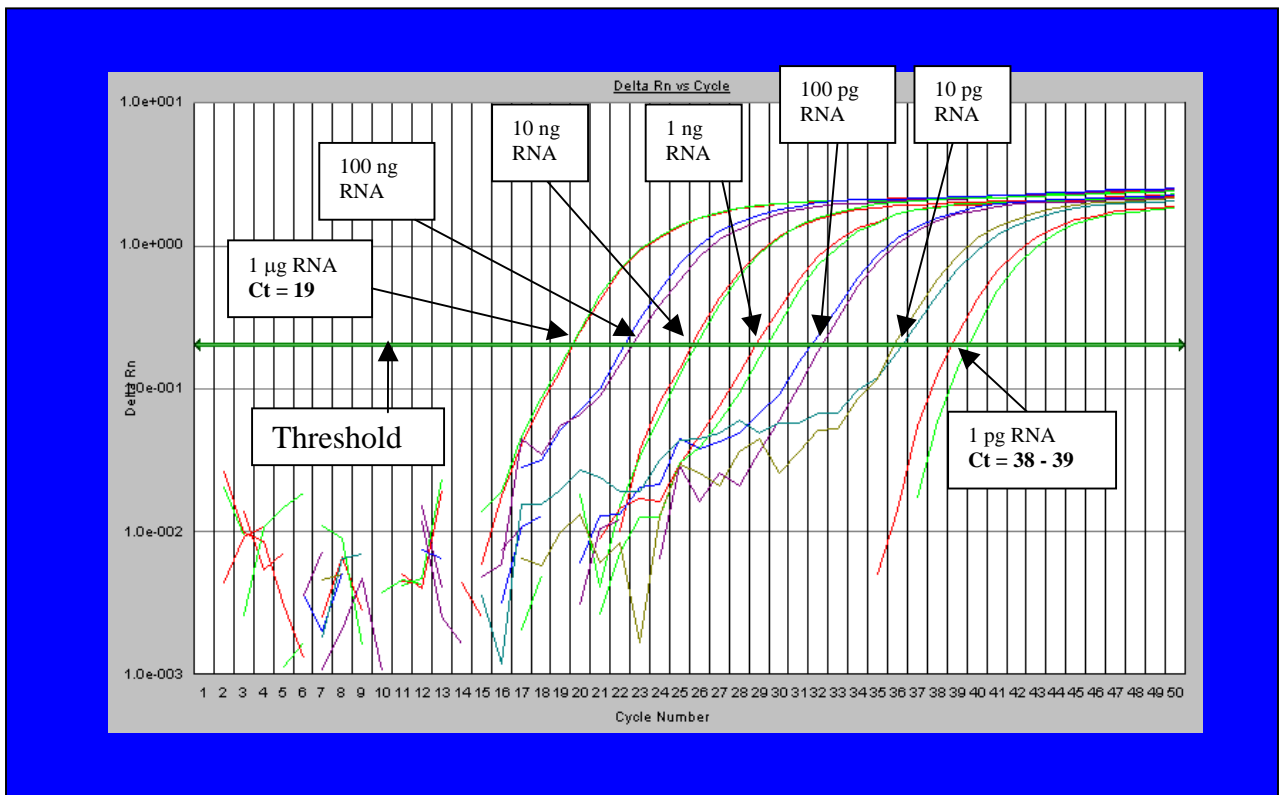
Finally, although the use of primer and gene-specific probe combinations increase the specificity of AD assays, it is a feature that also makes them more vulnerable to the misinterpretation of data in those cases in which unknown sequence alterations or polymorphisms are present in close vicinity to where these oligonucleotide sequences bind.

Gene Expression

Real-time reverse-transcription polymerase chain reaction is probably the most popular application for RQ-PCR and conversely, RQ-PCR is the most commonly employed method for the quantification of mRNA. Interestingly, this application of the technology is arguably the most complex and challenging. Why? The reason that RQ-PCR faces so many challenges in this area is simply that, one; every user follows a different set of experimental guidelines and two, in terms of RNA quality, “you only get out what you put in”. The source of many of the difficulties encountered is the lack of standardization. Every laboratory appears to have its own unique protocol, sample collection strategy, reagent management and quality control system, assay design, result interpretation and algorithm for data analysis. All of these contribute to the confusion often encountered by the novice user, but ultimately the major source of assay variability and poor data is the

integrity of the starting RNA template. RNA integrity is intimately related to the collection, shipping, storage, and extraction methods employed by the laboratory. In addition, the tissue source from which the RNA sample is extracted as well as the medical condition and therapeutic regimen of the patient being tested are likely to impact the integrity, if not, the differential expression levels of the target and reference gene sequences used in RQ-PCR quantification.

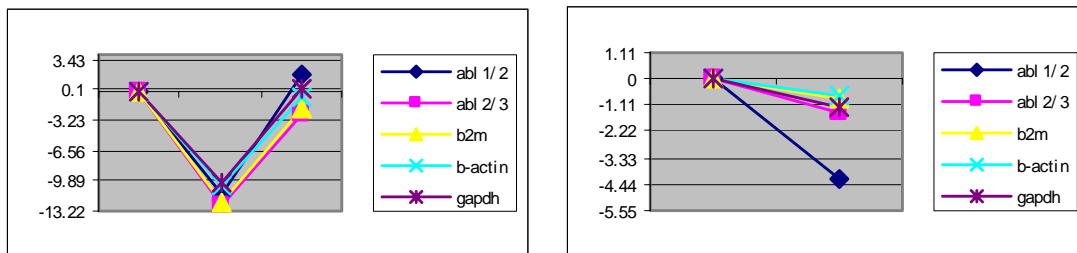
The two general approaches to nucleic acid quantification using RQ-PCR are absolute and relative quantification. In order to understand the general principles behind these methods, a brief description of the data collection process is required. As described by Ginzinger^v, the PCR reaction generates copies of a DNA template in an exponential fashion. Due to inhibitors of the polymerase reaction contained within the template, reagent limitations, and the accumulation of pyrophosphate molecules with time, eventually all PCR reactions will cease to generate amplicons at an exponential rate (“plateau phase”). Another outcome resulting from these factors is that some reactions will generate more end product than others, which is the predominant reason for why end-point quantification of PCR products is such an unreliable method. The ability to measure the PCR products as they are accumulating (real-time PCR), provides a measure of the amount of PCR product at a point in which the reaction is still within the exponential range. It is only during this exponential phase of the PCR reaction that it is possible to mathematically extrapolate the starting amount of template. During the exponential phase of a real-time PCR reaction, a fluorescence signal threshold (above which amplification is observed) can be determined as a function of the amount of background fluorescence. This signal output threshold is plotted at a point in which the signal generated from a sample is significantly greater than the background fluorescence. The fractional number of PCR cycles that a reaction requires to produce sufficient fluorescent signal to reach this threshold is known as the cycle threshold (Ct). These Ct values are directly proportional to the amount of starting template and serve as the basis for calculating mRNA expression levels or DNA copy number measurements. The diagram below shows a titration curve for beta-2-microglobulin (*B2M*) and highlights the threshold and Ct values. Notice that the reproducibility between duplicates is best when using high copy number targets.



Absolute quantification relies on the comparison of Ct values between an unknown sample and those of a standard curve. The curve can be generated using an internal calibrator or externally on a separate PCR well using a titration series of a well characterized template of known copy number. The objective of absolute quantification is to accurately quantify the presence of a target nucleic acid in a biological sample. Absolute quantification is a feasible alternative when the nucleic acid isolation procedure and sample contents have little or no impact on the PCR results. Quantification of stable genomic DNA may lend itself to absolute quantification against a titration curve of known standards. One challenge to this approach is how to ensure that the amplification kinetics of the known control or standards (typically a plasmid clone) closely mimic those of the genomic sample. The generation and independent quantification of known controls is difficult. In addition, maintaining stable and reliable control material is costly and time consuming as it requires thorough and periodic quality control measures. The major drawback to this approach though is that in gene expression experiments it fails to control for the integrity of the sample RNA material being tested. If one is not careful, variations in the isolation, handling, and RT-PCR components of an assay may lead to large deviations in the data when calculated against a standard curve.

In relative quantification, the objective is to make comparisons of nucleic acid quantities. This is typically performed by comparing the amplification kinetics (Ct values) of a target gene against those of an internal control or reference gene. In this manner, differences due to RNA integrity can be accounted for and Ct values can be normalized

between experiments. In addition, the Ct value of the reference gene can serve as a measure of both the quantity and quality of the input RNA used in the reaction. The challenges here include deciding on the method of choice for calculating the ratio of target to reference sequence, ensuring that the amplification efficiencies between the target and reference genes are comparable in every run, and most importantly understanding the influence that differential transcriptional regulation has on gene expression. The use of standard curves in conjunction with an internal reference gene and replicate testing provide the most conservative approach to the quantification of gene expression. Standard curves provide a measure of amplification efficiency for both the reference and target genes with each experiment and serve to monitor the dynamic range of detection for a particular target. One substantial challenge remaining is the task of defining the appropriate reference gene to use as part of the experimental design of an assay. Numerous studies have shown that the use of housekeeping genes may not be the answer as these are differentially regulated and may vary under experimental conditions^{vi}. These experimental conditions may include disease state, therapeutic regimens, or tissue source. Although the use of a reference gene under one set of experimental conditions may be adequate, it may be completely inappropriate to use that same gene for a different study. As illustrated in the diagrams below, the same five reference genes behave quite similarly in one patient sample (left), but one clearly appears as an outlier when tested on a different patient sample.^{vii} The graphs outline the change in expression of the *bcr-abl* fusion transcript starting at the time of CML diagnosis.



One final note of caution is that along with a broad detection range, the resolution of RQ-PCR is also quite broad. Therefore, in RNA expression assays, close attention must be employed in the interpretation of subtle changes in expression (below 10-fold). In using RQ-PCR for genomic DNA studies involving the loss of an allele (LOH, hemizyosity) or aneuploidy, it is extremely challenging to delineate thresholds between normal and affected tissues.

Conclusion

RQ-PCR is a powerful and multi-faceted technology platform with many applications in both the research and clinical laboratory settings. Although there are many advantages to using RQ-PCR over other methods, there are a number of limitations and challenges that the user must carefully evaluate as part of a comprehensive validation of any experimental and clinical protocol. These include an understanding of the chemistry, instrumentation, controls, QC, clinical application, sample requirements, data analysis and economics involved with using RQ-PCR in the laboratory.

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- ^{vii} Choppa P., et al. (2004). Evaluation of five control genes in the normalization of real-time quantitative PCR (QRT-PCR) data for residual disease monitoring in *BCR-ABL*-positive patients. *J. Mol. Diag.* **TT18**, 436.