The essential prerequisites for quantitative RT-PCR

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Quantitative application of the reverse transcription-polymerase chain reaction (RT-PCR) has been the subject of considerable debate for the last decade[1-5]. The goal of most quantitative RT-PCR methods is to use PCR product yield as a measure of relative differences in mRNA template abundance[1,6]. Because the efficiency of RT is usually assumed to be constant, the quantitative capacity of the PCR has been the primary focus of debate. Early on, the feasibility of quantitative PCR was questioned because of two theoretical constraints: (1) Given the exponential nature of the process, initially small tube-to-tube differences in amplification efficiency would grossly affect the final yield of PCR products[1-3]; and (2) PCR product yield could only provide a valid measure of template input during the exponential phase of amplification[2,4].

The development of competitive PCR methods allowed investigators to address such theoretical concerns and quantitative PCR was born[2,3]. In competitive PCR the final measure of template abundance is solely dependent on the initial ratio of target to competitor DNA templates. Thus, each reaction is internally controlled and theoretical arguments regarding the variables of amplification are largely irrelevant[3,5]. Given the initial skepticism, the strict internal controls of competitive PCR were essential to prove that quantitative PCR was *possible*. Since its development, however, few investigators have questioned whether or not competitive PCR methods are actually *necessary* to achieve a quantitative RT-PCR assay.

Competitive RT-PCR is often impractical for routine applications such as comparing the gene expression profile (i.e. multiple mRNAs) of large numbers of RNA samples[7]. Furthermore, when the primary source of variability is not the PCR, but the experimental system itself (e.g. environmental[6] or animal[7] studies), some investigators have questioned if precision should always take precedence over practical utility. Thus, alternative, high-throughput assays have been developed for comparing template abundance using conventional RT-PCR methods[6-10]. These "semi-quantitative" PCR methods are generally considered inferior, however, to competitive PCR because there is no internal control for amplification efficiency[1-5]. In the original description of competitive PCR, Gilliland et al.[3] provided evidence that inclusion of a competitor DNA was essential for accurate quantitation. Of three more recent side-by-side comparisons, however, competitive and "semi-quantitative" RT-PCR assays were found to produce equivalent measures of template abundance[9,11,12].

The assertion that competitor DNAs are essential to quantitative PCR is based on the original tenets that standard PCR amplification is (1) highly variable, and (2) proceeds to maximal levels in the plateau phase regardless of differences in template input. Although widely accepted as fact, the empirical evidence does not support either hypothesis. Following standard PCR amplification, a 10-20% coefficient of variability in product yield is typically observed from replicate DNA samples[8,9,13,14].

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Regarding the second point, the results of Halford et al.[14] demonstrate that PCR product yield in the plateau phase is not simply all or none. After 35 cycles of PCR, maximal amplification of PCR products occurs in all reactions containing greater than ~5 x 10^4 templates. However, as template becomes limiting (~5 x 10^2 - 5 x 10^4 templates), PCR product yield in the plateau phase is dependent on the logarithm of template DNA input[14]. Although the results are inconsistent with current PCR theory, Halford et al.[14] demonstrate that a major factor has been overlooked in past theoretical considerations: primer-dimers.

Despite optimal design, primers spontaneously form amplifiable primer-dimers at a low rate based on the sheer number of oligonucleotides in the PCR (~200 trillion per ml). Once formed, primer-dimers are efficiently amplified and serve as endogenous competitors of the PCR. As template concentration becomes limiting, primer-dimers constitute an increasing fraction of the total number of PCR products. When a reaction approaches the plateau phase, primer-dimers compete for reactants and thus inhibit the amplification of *specific* PCR products[14]. This phenomenon accounts for two related observations: (1) The lower limit of template detection in PCR run to the plateau phase is not the theoretical value of 1, but is normally 300–1000 templates per reaction[13,14], and (2) when template is limiting, PCR amplification reaches the plateau phase before specific PCR products accumulate to maximal levels (fig. 4 of ref. 1, fig. 3 of ref.14).

Ten years ago, the question was posed "What conditions must be satisfied to achieve a quantitative RT-PCR assay?" Based largely on theoretical considerations, the answer was that (1) each reaction had to be internally controlled by a competitor DNA[1-5] (2) the competitor DNA had to be nearly identical in sequence to the target DNA[3], and (3) product yields had to be measured in the exponential phase of amplification[2,4]. A decade later, none of these conditions have actually proven to be *necessary* for quantitative RT-PCR[5,9,14].

So the question remains "What are the essential prerequisites for establishing a quantitative RT-PCR assay?" To date, a great deal of effort has been spent defining the "correct" conditions that allow for quantitative RT-PCR. Given the inherent quantitative capacity of the method[14], however, perhaps it is time for RT-PCR to be treated like all other quantitative assays. That is, regardless of the specific PCR conditions chosen, the essential prerequisites for a quantitative RT-PCR assay should be that (1) a standard curve demonstrates the range over which PCR product yield provides a reliable measure of mRNA input, and (2) the number of samples tested allows for statistical analysis of differences in PCR product yield.

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