Comment on Wong and Medrano's "Real-time PCR for mRNA quantification" BioTechniques *39*:75-85 (July 2005)

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In a recent review, Drs. Wong and Medrano provided a good overview of the problems regarding mRNA quantification using real-time PCR (1). They claim that there are several pitfalls using this method for quantification and indeed it is, without a doubt, that fluctuation during PCR will affect quantification most since minute differences in PCR efficiency greatly affect the yield. However, as the authors also point out, the efficiency of the reverse transcription step is difficult to control, indicating that the reverse transcription step should be accounted for as well. It is a little bit surprising that Wong and Medrano draw the conclusion that if the cDNA synthesis step is directly coupled to the PCR step in one-tube "one-step RT-PCR," cRNA should be used as a control, while a two-step RT-PCR only requires DNA as a control. From my point of view, there is no difference between the two methods for converting mRNA into cDNA, meaning that both methods should use cRNA as a control. When quantitative PCR was introduced in the beginning of the 1990s, several groups used *internal synthetic standards* in the reactions to account for variances in reaction efficiencies, but these are not mentioned, suggesting that they have fallen out of fashion. These standards were typically identical to the target but lacked a sequence in the middle to be able to distinguish the standard from the target. Most importantly, they had the same primer sequences as the target, indicating simple multiplexing of the standard and the target. These internal standards can be used to absolutely quantify mRNA targets (2) and could solve many of the problems identified in the article by Wong and Medrano. It is easy to imagine such a standard in real-time PCR where the two PCR products (standard and target) could be separated by probes emitting different colors. Synthetic RNA internal standards have three advantages. First, by spiking the RNA sample with known amounts of standards, efficiencies in the reverse transcription step is accounted for. Since the standard is converted into cDNA, differences between tubes in the efficiency of the PCR are accounted for as well. Second, there is no need to worry about which reference gene to use for normalization. Third, absolute quantification could easily be obtained by relating to a *known* amount of internal standard (2) instead of relating to a relative internal standard, as in the case of a housekeeping gene and an external standard curve. It is not difficult to understand why such synthetic RNA standards are not used today since it takes some effort to produce and handle them without degradation, but it is difficult to understand why they are not considered as a solution or a tool for mRNA quantification using real-time PCR.

REFERENCES

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Response

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While it is true that a cRNA control can be used for both a one-step and two-step real-time PCR, we advocate the use of a DNA control for two-step reactions for two major reasons. First, they are much easier to synthesize and use with confidence than an RNA standard (i.e., they are less sensitive to degradation and thus more robust and reliable when used over a long period of time). Second, in our experience, many times a single cDNA synthesis reaction can be diluted and used for multiple different real-time PCRs. In many cases, one may wish to assay several different genes on a single sample, such as genes of interest plus housekeeping genes.

Consequently, using dilutions of a single cDNA synthesis reaction as the templates for the real-time PCR assays allows one to compare results across the different assays without factoring in different reverse transcription efficiencies, as all templates have the same reverse transcription efficiency. Granted, this method does not account for differences in reverse transcription efficiencies between different samples, but these differences should be controlled for with the use of normalization genes.

The "Comment" correctly points out that internal synthetic standards are a valuable tool for mRNA quantitation via real-time PCR. We did not mention these standards because, while they may be considered the "gold standard" in their ability to accurately control for differences in reaction efficiencies, they are not very practical for the average user.

These standards are difficult to produce, are prone to degradation, and while they can control for variation in reaction efficiencies, they cannot control for differences in the quality of template RNA; this variable must still be accounted for through the use of normalization genes. A current important area of real-time PCR application is the validation of gene

expression levels of large numbers of gene targets from microarray experiments. The inclusion of individual internal standards for large-scale experiments would be completely impractical.

In addition, we would also like to clarify that for the LUX™ primer technology from Invitrogen, it is possible to run a melting curve to examine any erroneous amplification since the fluorescence stays attached to the PCR product. This melting curve is run under the same conditions that one would use for a SYBR® Green I melting curve. Therefore, the presence of a single PCR product would likely be detected without the need to run an agarose gel, contrary to the statement in our original paper.

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Correction: Facile whole-body imaging of internal fluorescent tumors in mice with an LED flashlight

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An error appeared in Yang et al. (*BioTechniques 39:170-172, August 2005*; doi 10.2144/000112093) in the article entitled "Facile whole-body imaging of internal fluorescent tumors in mice with an LED flashlight." On page 170, paragraph 4 should read as follows:

It is reported here that a blue LED flashlight (LDP LLC, Woodcliff Lake, NJ, USA; www.maxmax.com/OpticalProducts.htm) with the excitation D470/40× filter (midpoint wavelength peak of 470 nm) and the OG515 Long Pass emission filter (Chroma Technology, Rockingham, VT, USA) for viewing could be used for whole-body imaging of mice with GFP and red fluorescent protein (RFP)-expressing tumors growing in or on internal organs (2).

The authors regret these oversights. The corrected PDF version of the article is available at www.BioTechniques.com.