Does Pipetting Error Affect the Consistency of PCR Array Results?

Introduction

It is a commonly held belief that the reproducibility of RT-PCR results is highly influenced by pipetting variation. This notion is intuitive and correct, but is successfully addressed when using a master mix containing a reference dye with the RT²*Profiler*™ PCR Array. Thoughtful researchers have pointed out that the multi-channel pipettors recommended in the PCR Array protocol are convenient, but imprecise. A typical concern is that the error inherent in the use of these pipettors will exacerbate the variability of raw C_t values and the gene expression fold-changes, thereby undermining the value of analyzing multiple genes simultaneously. This Technical Note demonstrates that pipetting error does not affect the determination of C_t values on the RT²*Profiler*™ PCR Arrays when analyzed on instruments that use a reference dye.

Materials and Methods

The RT² Real-Time™ Human ACTB qPCR Primer Set (PPH00073A) was aliquoted and dried into each well of a 96-well PCR plate. XpressRef™ Universal Human RNA (1 µg, GA-004) was converted to cDNA using the ReactionReady™ First Strand cDNA Synthesis Kit (C-01). The cDNA was then mixed with the RT² Real-Time™ SYBR Green / ROX PCR Master Mix (PA-012) as described in the PCR Array User Manual. Various volumes of the cocktail (15, 20, 22.5, 25, 27.5, or 30 µl) were added to a triplicate set of wells using a multi-channel pipettor. PCR was performed on the ABI 7500 FAST instrument. Threshold cycle values were determined with SYBR Green as the detector dye, with or without ROX as the reference dye. Default or manually set thresholds were used with or without the reference dye, respectively.

Reference Dyes Normalize Pipetting Error

Figure 1 illustrates the effect of pipetting error on PCR Array results obtained with the use of a reference dye. The pipetting error purposefully implemented in this experiment from the recommended 25 µl volume ranges from -40 to +20 percent. However, the threshold cycle value determined at each volume is virtually identical (Figure 1A) with differences from the C_t at the correct volume within only ± 0.05 cycles, or one-third of one percent, (Figure 1B). The variation between the replicates at each volume was negligible, with an average standard deviation of only 0.031 cycles. This variation is more representative of true pipetting error. (The sizes of standard deviation error bars in Figures 1A and 1B are smaller than the graph symbols, while the y-axis of the Figure 1B inset is expanded to visualize the small magnitude of the variation.)

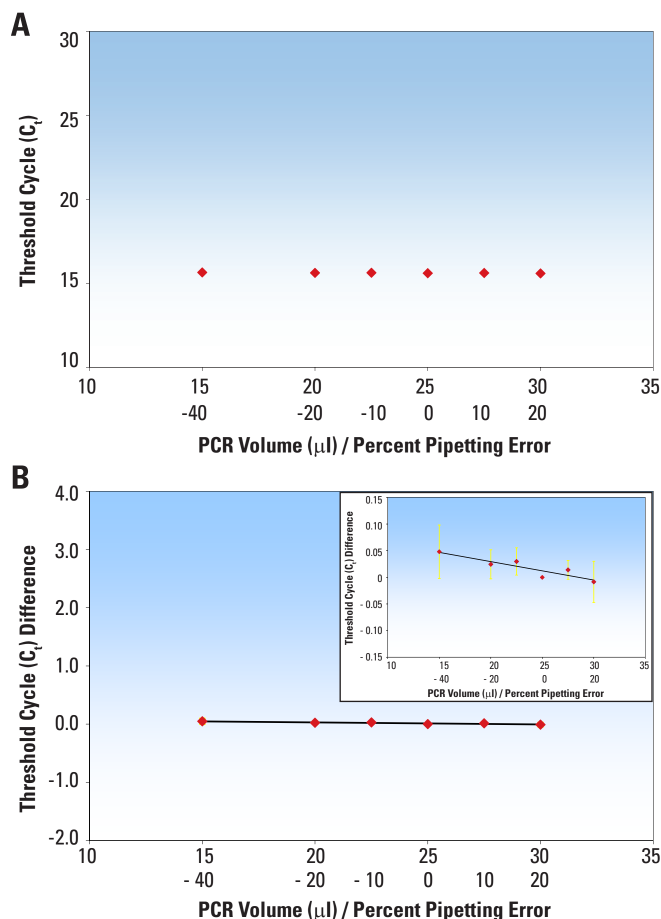

Figure 1: Reference Dyes Eliminate Pipetting Error Effects on C_t Values. Panel A displays the threshold cycle of ACTB determined using different volumes of template and master mix cocktail. Panel B displays the difference in ACTB threshold cycle value at each volume relative to the correct one (25 µl). The inset of Figure 1B expands the y-axis to visualize the error bars that represent one standard deviation from the mean values.

We previously found that the average standard deviation in C_t values for technical replicates on the PCR Array is roughly 0.25 cycles (Pathways, Issue 2, Fall 2005). The exaggerated pipetting error causes much smaller C_t value differences, while the variation associated with normal pipetting error is even smaller. Therefore, pipetting error contributes very little to the overall variability of the PCR Array method when using a reference dye in the master mix and in data analysis.

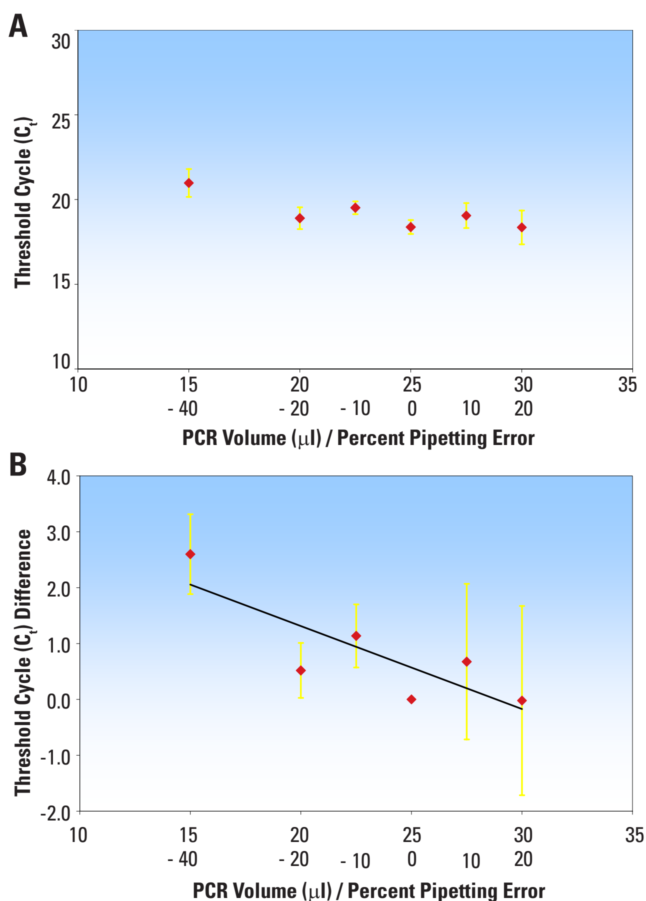

Figure 2: Pipetting Error Dramatically Affects C_t Value Determination in the Absence of a Reference Dye. Panel A displays the threshold cycle of ACTB determined using different volumes of template and master mix cocktail. Panel B displays the difference in ACTB threshold cycle value at each volume relative to the correct one (25 µl). The error bars in both panels represent one standard deviation from the mean values.

Pipetting Error Apparent Without Reference Dye

The data analysis in Figure 1 relied on the ROX reference dye utilized by the ABI instrumentation. Stratagene instruments can also use ROX as a reference dye, while Bio-Rad instruments use fluorescein. We expect the effects of pipetting error on the C_t value determination using these other instruments to be similarly small. However, not all real-time PCR instruments utilize a reference dye. To estimate the effects of pipetting error on the consistency of the PCR Array results without a reference dye, the amplification plots used to acquire the results in Figure 1 were re-analyzed without ROX (Figure 2).

With a large pipetting error (25-µl, -40 to +20 percent), the threshold cycle value varies widely without a reference dye (Figure 2A). The differences from the C_t at the correct volume are as great as ± 2.5 cycles or 32 percent (Figure 2B). The actual pipetting error, or the variation between the replicates seen at any given volume, is also much larger without the reference dye. The average standard deviation is 0.664 cycles and is clearly visible on the scale of both representations of the data in Figure 2.

The C_t value variation due to pipetting error is much greater than the average standard deviation in C_t values for technical replicates on the PCR Array (our reproducibility assessment of 0.25 cycles). Thus, without the reference dye to correct it, pipetting error contributes significantly to the overall variability of the method and makes smaller fold-changes in gene expression very difficult to detect.

Conclusions

The results presented in this Technical Note illustrate that normal pipetting error does not affect threshold cycle value determinations on real-time PCR instruments that use a reference dye. On such instruments, the PCR Array protocol's reliance on repeat pipetting using a multi-channel pipettor does not significantly influence its results. A constant reference dye concentration across the plate provides a signal proportional to the actual volume in the wells. By normalizing SYBR Green signals with the reference dye, the instrument software also normalizes for differences in well volume. Instruments without a reference dye cannot correct for pipetting error, and so it becomes imperative to consistently dispense equal volumes of the master mix and template cocktail into the PCR Array.

Recommendations

Follow the typical pipetting rules and precautions to control pipetting error. Electronic pipetting is preferable to manual pipetting, if available. Once the solution is drawn, be sure that you do not carry over any excess volume on the outside of the tip. Use fresh tips each time solution is dispensed. It is always important to keep in mind that performing replicate PCR Arrays will ascertain the real level of variation in individual experiments.

RT² Real-Time™ PCR Master Mixes

Find the qPCR master mix for the instrument in your lab

Instrument Type	Detection	Optimized Master Mix	Cat. No.
Applied Biosystems	SYBR Green	RT² Real-Time™ SYBR Green/ ROX	PA-112
and Stratagene	TaqMan	RT² Real-Time™ ROX	PA-114
Bio-Rad	SYBR Green	RT² Real-Time™ SYBR Green/ Fluorescein	PA-111
Other Brands	SYBR Green	RT² Real-Time™ SYBR Green	PA-110