SLOPE-INTERCEPT ADJUSTMENT FORMULA TO OBTAIN CONSISTENT RT-PCR QUANTIFICATION RESULTS

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The North Louisiana Criminalistics Laboratory DNA Unit began using the Quantifiler® Human and Quantifiler® Y Kits (Applied Biosystems) in late 2004. Initially, during the validation of the ABI Prism® 7000 Sequence Detection System (SDS) and the two quantification kits, the amplicon peak heights were usually well below the maximum RFU threshold on the electrophoresis units and seldom exhibited pull up or other artifacts caused by too much DNA. However, the average peaks heights often varied considerably between amplifications and sometimes were too low causing a loss of sensitivity. A fixed adjustment factor was initially recommended to reduce the variance of the peak heights, but the resulting peak heights were still inconsistent and began to demonstrate off-scale peaks. A robust adjustment factor was sought which would fully utilize the dynamic range of the electrophoresis units and produce consistent results between runs.

Real-time PCR (RT-PCR) instruments produce a standard curve to estimate the DNA concentration ([DNA]) in samples. Two variables, slope and y-intercept, are used to calculate this standard curve. The slope represents the ratio of the scales of the y-axis and x-axis, and the y-intercept represents the cycle threshold (CT) value for 1.0 ng/µL of DNA, which ABI reported had a value of ~28.5. Of the two variables, the CT has the greatest effect on the results because a difference of ±1 alters the [DNA] by a factor of 2. The extreme differences in quantification results we encountered seemed to correlate with the CT value calculated from the standards.

Three potential adjustment formulas using the slope and intercept values were considered in the development of a robust adjustment factor: (1) slope-only; (2) intercept-only; and (3) slope-intercept combined. Using the results of previous amplifications an ideal slope and ideal CT value were determined for use in the three adjustment formulas. These formulas were then used to adjust the quantification results of a serially-diluted NIST traceable sample (2.0 ng/µL). The unadjusted and slope-only adjusted [DNA] quantified at three times the expected value, which resulted in a large overestimation of the [DNA]. At all [DNA], the intercept-only and slope-intercept adjustments estimated the [DNA] to within 0.01 ng/µL of the expected value.

Run-to-run and analyst-to-analyst reproducibility were evaluated using the DNA quantification results from five analysts, who were given ten samples for their competency test. The unadjusted results demonstrated an average standard deviation of 1.86, and the values uniformly overestimated the [DNA]. After the slope-intercept adjustment, the average standard deviation was 0.99, and all of the values were at or slightly below the expected concentrations of the competency samples. Lot number-to-lot number variance was evaluated by running paired samples with the old and new lot numbers over several series of Quantifiler® Human lots. Without adjustment the mean difference in the [DNA] of the paired samples was 0.345 ng/µL (SD=0.402), but after adjusting the [DNA] with the slope-intercept formula, the mean difference was 0.000 (SD=0.009). In further experiments, samples calculated from standard curves, which had outliers causing an R^2 value < 0.98, were re-evaluated with the slope-intercept formula and compared to the results after removing the outliers and recalculating the standard curve ($R^2 > 0.99$). The mean difference in the paired samples changed from -0.068 (unadjusted, SD=0.247) to -0.007 (slope-intercept, SD=0.014).

The slope-intercept equation has been shown to be a robust adjustment factor for estimating DNA concentration analyzed by the ABI Quantifiler® Kits. The adjustment factor has been successfully used with the AmpFlSTR® Series of kits (Profiler®, Profiler Plus®, COfiler®, SGM Plus®, Identifiler®, MiniFiler™, and Yfiler™) and the Promega PowerPlex® 16 Kit. The formula has been used with the

following RT-PCR instruments: ABI 7000 SDS, QIAGEN Rotor-Gene® Q, and the Roche LightCycler® 480. The procedure has worked with manual pipetting and two liquid handlers (QIAGEN QIAgility™ and PerkinElmer JANUS Mini). The research presented here reports on a robust slope-intercept formula which can be used to adjust quantification data from RT-PCR instruments to insure a full range of amplification. The formula offers excellent run-to-run, kit-to-kit, and analyst-to-analyst reproducibility and will correct for standard curves with less than optimum (<0.98) R^2 values.