Original Research Article

Improving qPCR Telomere Length Assays: Controlling for Well Position Effects Increases Statistical Power

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Objectives: Telomere length (TL) is commonly measured using quantitative PCR (qPCR). Although, easier than the southern blot of terminal restriction fragments (TRF) TL measurement method, one drawback of qPCR is that it introduces greater measurement error and thus reduces the statistical power of analyses. To address a potential source of measurement error, we consider the effect of well position on qPCR TL measurements.

Methods: qPCR TL data from 3,638 people run on a Bio-Rad iCycler iQ are reanalyzed here. To evaluate measurement validity, correspondence with TRF, age, and between mother and offspring are examined.

Results: First, we present evidence for systematic variation in qPCR TL measurements in relation to thermocycler well position. Controlling for these well-position effects consistently improves measurement validity and yields estimated improvements in statistical power equivalent to increasing sample sizes by 16%. We additionally evaluated the linearity of the relationships between telomere and single copy gene control amplicons and between qPCR and TRF measures. We find that, unlike some previous reports, our data exhibit linear relationships. We introduce the standard error in percent, a superior method for quantifying measurement error as compared to the commonly used coefficient of variation. Using this measure, we find that excluding samples with high measurement error does not improve measurement validity in our study.

Conclusions: Future studies using block-based thermocyclers should consider well position effects. Since additional information can be gleaned from well position corrections, rerunning analyses of previous results with well position correction could serve as an independent test of the validity of these results. Am. J. Hum. Biol. 00:000–000, 2015.

Telomeres are repetitive DNA sequences (5′-$[TTAGGG]_n$-3′ in vertebrates) that cap both ends of linear chromosomes (Blackburn and Gall, 1978; Meyne et al., 1989). Telomere length (TL) is implicated as a determinant of senescence, immune function, cancer, and other traits (Eisenberg, 2011) and in recent years has become a frequently measured biomarker in human and nonhuman studies.

The southern blot analysis of terminal restriction fragments (TRF) method of TL measurement was the first widely accepted technique used (Moyzis et al., 1988), and continues to be considered the "gold standard" TL measurement method (Aubert et al., 2012). More recently, qPCR based methods have become the most widely used means of TL measurement (Cawthon, 2002, 2009). The qPCR method uses creatively designed primers with intentional sequence mismatches to amplify the telomere repeat sequence without primer dimer formation. The qPCR method is standardized to a "single-copy-gene" control (similar to a "housekeeping" gene control in expression analysis). The qPCR method requires roughly 1/50th the DNA of the TRF method and is considerably less expensive and time consuming (Aubert et al., 2012; Turner et al., 2014). Although more efficient, qPCR method results tend to have higher measurement error and yield less consistent results across laboratories when compared to TRF (Eisenberg, 2014; Gardner et al., 2014; Haycock et al., 2014; Martin-Ruiz et al., 2014). For example, some common associations, such as the finding of shorter TL among males, are evident in some qPCR studies but not others, while TRF findings are more consistent (Gardner et al., 2014). This heterogeneity might result from increased measurement error in some laboratories limiting the statistical power to detect associations. There are many potential sources of differential error across laboratories including differences due to DNA extraction techniques (Boardman et al., 2014; Cunningham et al., 2013; Denham et al., 2014; Hofmann et al., 2014), dilution of DNA, primer choices, mastermix choices, pipetting errors, thermocycler, and data analysis strategies. Here we explore a likely candidate source of measurement error: well position effects. Controlling for these well position effect might help to minimize measurement error and account for the observed heterogeneity of associations in studies.

Our preliminary experiments across several block based thermocyclers (Bio-Rad iCycler iQ, Bio-Rad MyiQ, Bio-Rad

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Additional Supporting Information may be found in the online version of this article.

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CFX 384, Roche LightCycler 480, MJ Research Chromo 4, and Eppendorf MasterCycler ep realplex) have shown systematic variation in estimated concentrations depending on well position on the thermocycler block. We are not aware of previous studies directly addressing this well position issue on TL measurement, although the concern has been implicitly acknowledged through the randomization of well positions across replicates in previous studies (e.g., Cawthon, 2009; Drury et al., 2014).

Here we seek to evaluate the importance of well position effects by first characterizing the degree of heterogeneity across wells, correcting for this heterogeneity, and then examining how the correction affects performance. To accomplish this, we reanalyze previously published (Eisenberg et al., 2012) qPCR based TL measurement data from over 3,000 individuals run using a modified version of the monochrome multiplex quantitative polymerase chain reaction (MMQPCR) protocol (Cawthon, 2009). To evaluate whether correcting for well position effects improves assay performance we use three validation measures known to be strongly correlated with TL. Since random measurement error causes an attenuation of associations (Greene, 2008, p. 325), methods that minimize measurement error will tend to increase the strength of associations and thereby increase statistical power. By using three distinct validation measures we gain convergent validity and lessen the probability of being misled by chance findings. First, we use TRF measures obtained on a subset of 190 individuals to compare unadjusted qPCR TL correlations with TRF to well-adjusted qPCR TL correlations with TRF. Second, we examine the strength of the established negative correlation between TL and age ($n = 3{,}638$)—again comparing unadjusted and well-adjusted correlations. Finally, since TL is highly heritable, we examine the mother-offspring correlation in TL ($n = 1{,}500$ mother-offspring pairs).

We also examine three other additional aspects of TL data analysis methods that could influence performance. First, since a previous large study found a nonlinear relationship between telomere (T) and single-copy-gene (S) amplicons (Kvale et al., 2012), we examine whether such a nonlinear relationship is present in our data. Second, some, but not all, studies examining the association between qPCR and TRF measurements have shown non-linear associations (Elbers et al., 2013). We evaluate whether a quadratic fit between qPCR and TRF measurements better fits the data than a linear fit. Third, many telomere studies exclude samples with coefficients of variation (CV) across replicates greater than a particular threshold value (often 10% or 15%) in an effort to eliminate noisy samples (i.e., samples with low precision), which may reduce statistical power. We note that the CV is not an ideal statistic of measurement error because it is invariant to the number of replicate measurements. We examine how variable exclusion thresholds using standard error in percent of the mean (standard error/mean $\times$ 100; SE%) affect assay performance. SE%, unlike CV, decreases with increasing numbers of replicates, better reflecting the precision of the estimate.

MATERIAL AND METHODS

Samples and data

Samples and data are from the Cebu Longitudinal Health and Nutrition Survey (CLHNS), a birth cohort study in Cebu City, Philippines that began with enrollment of 3,327 pregnant mothers in 1983–1984 (Adair et al., 2011). Venous blood samples were collected in 2005, when the offspring were 20.8- to 22.5-years-old and the mothers were 35.7- to 69.3-years-old. Automated and manual DNA extraction (Puregene, Gentra) was conducted on venous blood from 1,893 mothers and 1,779 offspring. Written informed consent was obtained from all participants and data and DNA collection were conducted with approval and oversight from the Institutional Review Boards of University of North Carolina and Northwestern University. Telomere measurement and analysis in deidentified samples and data was not considered human subjects research by Northwestern University's Institutional Review Board.

Telomere length measurement

qPCR, TLs were measured using the MMQPCR assay (2009) with the following modifications as previously described (Eisenberg et al., 2012). Reactions were run with telomere primers (telg/telc) at 500 nM each and albumin (single-copy control) primers (albd/albu) at 300 nM each on a Bio-Rad iCycler iQ thermocycler with a modified thermo-profile: internal well factor collection for 1.5 min at 95°C, denaturation and Taq activation for 13.5 m at 95°C, 2 repeats of: 2 s at 98°C followed by 30 s at 49°C, 34 repeats of: 2 s at 98°C, 30 s at 59°C, 15 s at 74°C with signal acquisition, 30 s at 84°C, 15 s at 85°C with signal acquisition, followed by a melt curve for PCR product verification. Data were analyzed with a per-well efficiency calculation method (Ehrlenbach et al., 2009, 2010; Willeit et al., 2010) using LinRegPCR version 12.7 (Ramakers et al., 2003; Ruijter et al., 2009). All T/S ratios were normalized to (divided by) the T/S value of the same control sample run with six replicates per 96 well plate. Previous experiments with the Bio-Rad control assay and this TL assay revealed that corner wells of the PCR plate had consistent and substantially discrepant results from other wells (this is a common problem to Bio-Rad iCycler and Bio-Rad CFX 384 well platforms operating within company specifications, and probably a more general problem with peltier based thermocyclers; Herrmann et al., 2007). Therefore, only negative controls were placed in two of the corner wells (Row H), while corner wells in Row A were left empty. More detailed information on the qPCR assay implementation is given as Supporting Information 1.

Southern blot TRF

Southern blot analysis of TRF was performed using the restriction enzymes HinfI and RsaI after verification of DNA integrity (Kimura et al., 2010). One-hundred and ninety samples were measured, and 159 of these samples measured twice, and averaged and the remainder measured only once. Of those 159 samples measured twice, the average SE% was 1.53% (equivalent to a CV in percent of 2.17%).

RESULTS

Well position

To examine how well position effects our MMQPCR results, first we calculated the mean T/S value in each well position across all 180 available runs. Each well position contained an average of 174.8 replicate values (range: 123–180). Figure 1 depicts this variation graphically and notes that the CV% of average values across the thermocycler block is 8%. As is apparent visually, T/S values tend

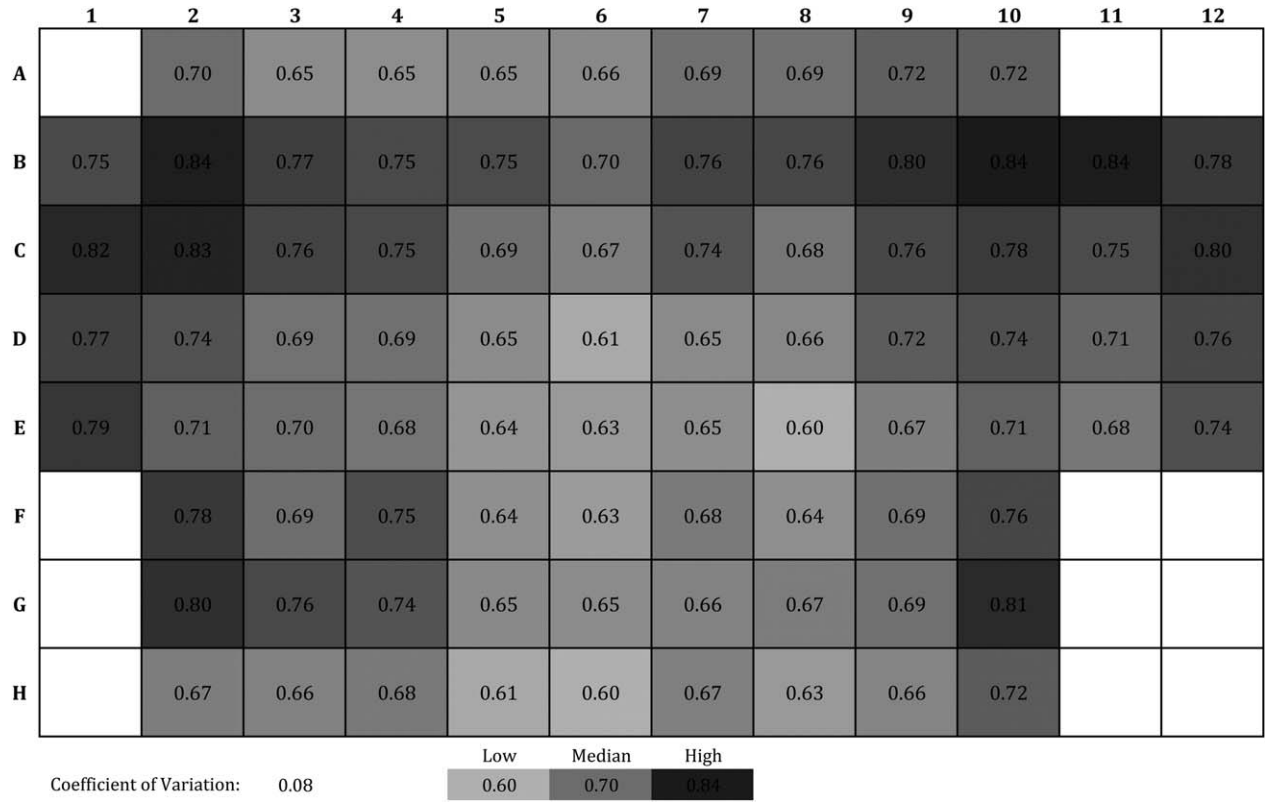

Fig. 1. Average qPCR telomere length measures by well across 180 qPCR runs. Wells A1, A11, and A12 contained no sample, control samples were in wells F1, F11, F12, G1, G11, G12, negative water controls in H1, H11, and H12 and all other wells contained experimental samples.

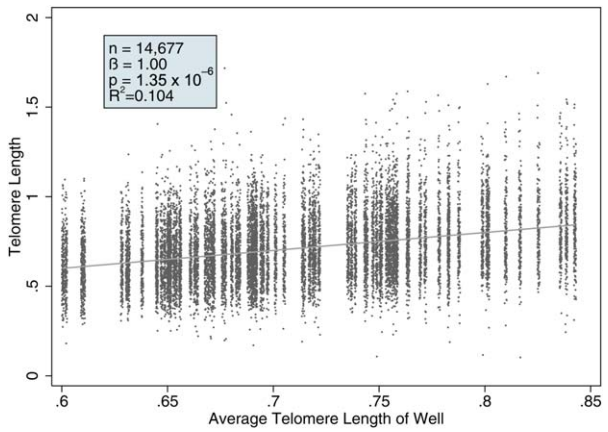

Fig. 2. Average telomere length of each well position (from Fig. 1) predicts each well specific telomere length measure. 10.4% of the variation in T/S values is attributable to well position.

to be lower closer to the center of the plate. In multivariate regression with T/S value as the outcome and number of wells distance from the closest X-axis edge (left or right, 0–6 wells) and Y-axis edge (top or bottom, 0–4 wells) as predictors, being one well closer to an X-axis edge predicted a 0.0247 unit increase in T/S values ($P < 0.001$) while distance from Y-axis edge was not a significant predictor ($\beta = 0.0033$, $P = 0.468$) and the overall adjusted R^2 was 0.41. To analyze how well position affects individual well T/S values, we examined the degree to which the average T/S of each well position (Fig. 1) predicted each T/S value. The average T/S of each well position (shown in Fig. 1) predicted 10.4% of the variation of the individual well T/S values (n = 14,677 wells; Fig. 2). This suggests that 10.4% of the variation in T/S values is attributable to our measure of well position (and the remaining 89.6% to true TL variation across samples and other sources of error).

To control for these well position effects, every well specific T/S value was adjusted by subtracting the well position specific mean value (i.e., values in Fig. 1) and adding the overall mean T/S value (overall mean T/S across all 14,677 wells). Unadjusted sample mean T/S values and well position adjusted sample mean T/S values were then related to our three validation measures. Across all three measures, correlations consistently increased in magnitude (Table 1).

To quantify the estimated power gains yielded from controlling for well position effects (Table 1) we compared the sample sizes needed to distinguish unadjusted versus well-adjusted qPCR TL correlates as significantly different from zero ($\alpha = 0.05$, power = 0.95.) For example, to detect the correlation of qPCR TL with TRF TL without well-position adjustment ($r = 0.637$) would require a sample size of 25.91, while a sample of 23.40 is required to detect the well-position corrected correlation coefficient ($r = 0.663$; power calculated using Stata sampsi_rho command). This equates to an 11% increase in sample size. Using the same procedure for age and mother-offspring correlations yields estimated increases in sample sizes of

TABLE 1. Changes in correlation coefficients with measures of external validity before and after well-position correction

	Correlation coefficients		
	Unadjusted qPCR TL	Well-adjusted qPCR TL	Difference
TRF (n = 190)	0.637	0.663	0.026
Age (n = 3,638)	−0.416	−0.436	0.020
Mother–Offspring (n = 1,500)	0.258	0.290	0.033

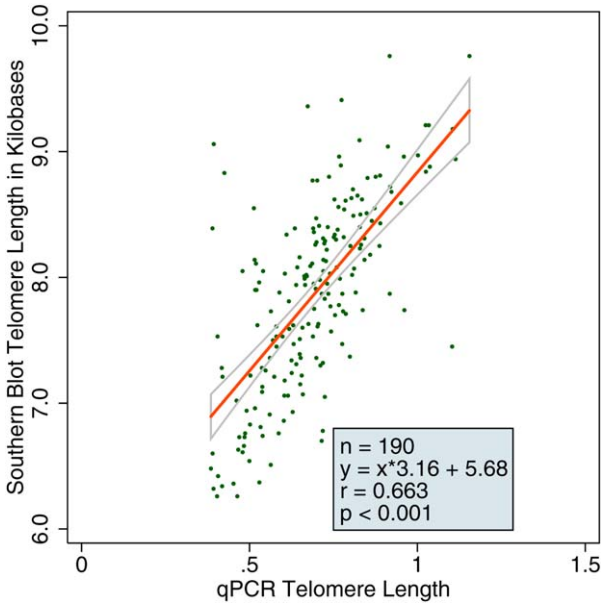

Fig. 3. qPCR versus Southern Blot telomere length measurements. No quadratic association is evident (quadratic term $P = 0.703$).

11% and 27%, respectively. Averaging these three estimates suggests that the well position correction yields a gain in power equivalent to a 16% increase in sample size.

Nonlinearity

Since a previous large scale analysis found a nonlinear relationship between telomere (T) and single-copy-gene (S) amplicons (Kvale et al., 2012), we examine whether such a nonlinear relationship is present in our data. By including a quadratic term in an OLS regression model with T as the outcome, no deviations from linearity were detected (quadratic term P = 0.894).

Some previous studies have also found a nonlinear relationship between qPCR and TRF measurements (Elbers et al., 2013). As with T/S above, we tested this by including a quadratic term in an OLS regression model with TRF as the outcome (Fig. 3). Again, no quadratic association is evident in our data (quadratic term P = 0.703). We note that the correlation of these 190 samples with age using MMQPCR is −0.508 (P < 0.0001) and using TRF −0.456 (P < 0.0001), suggesting that for this measure of assay validity our MMQPCR might perform better than TRF

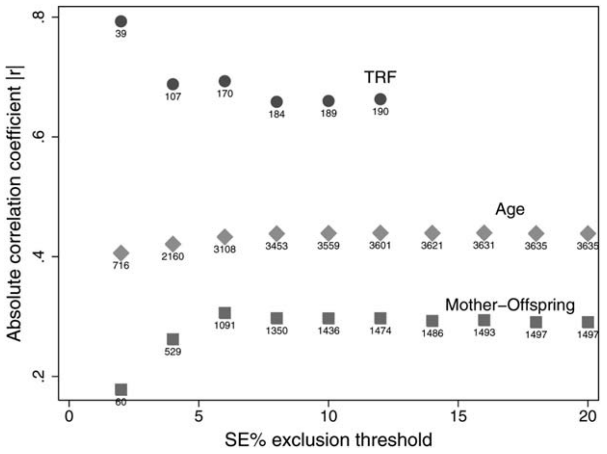

Fig. 4. Correlation coefficients between TL and validation measures in relation to varying the SE% exclusion cut points. Circles represent correlations of qPCR measures with TRF, diamonds of qPCR measures with age and squares of mother-offspring qPCR TL correlations. Numbers below each symbol note sample size.

(but P = 0.51 of difference in these two correlation coefficients).

Coefficient of variation and handling "noisy" samples

Using the SE% measure (standard error/mean × 100), we experimentally varied our exclusion criteria in an effort to find an optimum exclusion threshold. As noted above, the SE% measure is a better measure than CV because it decreases with increasing replicates to reflect the increasing precision of the estimate with increasing replicates. Well-adjusted qPCR mean TL were excluded if SE% was greater than 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20% in an effort to find an exclusion point at which correlations were consistently higher across the three measures. For comparison, we note that the commonly used CV% exclusion criteria of 15% and 10% with three replicates is equivalent to a SE% of 8.66% and 5.77%, respectively. As shown in Figure 4, no consistent optimal cut point is apparent nor is there a consistent directional relationship between the exclusion point and correlation magnitudes. For example, there is no meaningful difference in the correlation coefficient measured when using a threshold of >6 SE% and >20 SE%. However, using the more restrictive (i.e., lower SE%) threshold decreases the number of samples (up to 27% in the mother-offspring case). Further, correlation coefficients vary little between the 20% SE% threshold and no exclusion at all (values in Table 1).

DISCUSSION

Our analyses of MMQPCR results from over 3,000 human DNA samples across 180 qPCR runs on 96 well plates shows substantial well position effects. Correcting for these well position effects resulted in consistently improved measurement validity. Controlling for well position effects improves statistical power equivalent to an estimated 16% increase in sample size. Because our preliminary analyses had indicated more severe well position effects in corner wells, no samples analyzed here were placed in these wells. If samples had been placed in corner

wells, we expect that well correction would have yielded an even larger improvement in measurement validity than what we report here.

The increase in statistical power equivalent to a 16% increase in sample size that we report with well position-correction represents a substantial reduction in the needed sample, and thus costs and participant burden, to conduct telomere-based research. The importance of well position correction and concomitant power gains is even more important in the case of small isolated populations and historically banked biological specimens where it may not be possible to increase sample size. If this method of well position correction also applies to data from other laboratories, this equates to a large untapped set of data. Not only will future analyses benefit, but by redoing previous analyses with well correction, new data is effectively derived from these existing data, allowing pseudo-replications of previous findings for trivial costs. If previously observed unadjusted associations between TL and other variables become stronger with well position adjustment, this would suggest that the association in question is more likely to be correct (and vice versa).

We note that while these results provide verification of our MMQPCR measurements and recommendations for future analytical strategies, results may vary across instruments and laboratories. Adjustments for well position effects need to be made for each particular instrument (i.e., well position effects should not be assumed to be the same for even the same make and model of instrument without first verifying). We also stress that external validity measures need to be measures that are not the intended focus of study. Our analytical strategy systematically seeks higher associations with these external variables, so the estimated correlations with these variables may be over-inflated. For example, in our study after the implementation of well-position corrections based in part upon the strength of association between TL and age, it would now be dubious to compare the qPCR measured age related decline in our population with other populations (although our standardizing to three different external measures mitigates this concern). But, if a study focuses on the relationship between TL and disease status, using the association between TL and age would be an appropriate validation.

In additional analyses of our MMQPCR data, we show that there is a linear relationship between telomere (T) and single-copy-gene (S) amplicons, that our MMQPCR measures have a linear relationship with TRF and that excluding noisy samples (i.e., high SE% samples) does not improve measurements. While it is unclear why we did not find nonlinear relationships like some previous studies, one possibility is that there are genetic polymorphisms (e.g., copy number variants and/or SNPs) in some of the single copy gene controls used (there are several in use in the literature) in some studies, or specific to some populations, which cause samples with high or low S values to have only average T values. The single-copy control primer pair in the albumin gene was chosen for this study because we observed fewer polymorphisms in the corresponding genomic sequence, according to online databases and SNPbrowser 4.0 (Applied Biosystems), than the beta-globin primers also tested in the MMQPCR protocol. To test for this possible influence of single copy amplicon choice influencing linearity, future studies which show a non-linear association between T and S could examine whether the same pattern is shown for other single-copy controls run on the same samples. Nonlinear associations between T and S due to genetic polymorphisms in S might also lead to nonlinear relationships between uncorrected T/S values and TRF values.

Alternative thermocycler technologies may eventually minimize or eliminate altogether the well position effects reported here. Peltier element based thermocycler blocks are the dominant technology employed for qPCR, but are thought to lead to limitations in temperature uniformity across the block (Javorschi-Miller and Orlic, 2011), which might explain the well position effects observed here. We are aware of two alternatives to Peltier based thermocyclers which have considerably improved temperature uniformity and which might therefore help eliminate systematic well position effects on the qPCR telomere assay: (1) rotary real-time PCR thermocyclers, which spin samples in a centrifuge and cycle temperatures with air cooling and heating and (2) a thermocycler block filled with a thermal conductive fluid that is mixed with electromagnetic paddles to improve uniformity (Javorschi-Miller and Orlic, 2011). The highest throughput version of the rotary thermocycler, the Qiagen Rotor-Gene Q, can accommodate up to 100 wells/run. Only one thermocycler, the Illumina Eco, has a block containing thermal conductive fluid. However, the Eco has been discontinued and only had a block size of 48 wells. While the Rotor-Gene Q appears as the obvious choice to eliminate well-position effects, the well-to-well variation of this platform has not been investigated and a recent comparison of samples measured using TRF and singleplex qPCR measured TL on the Rotor-Gene Q found correlations between TRF-qPCR slightly lower ($P = 0.077$ for differences in correlation coefficients) than found in this study (Gutierrez-Rodrigues et al., 2014), suggesting that other sources of variation beyond well position effects might emerge in some implementations on the Rotor-Gene Q. While the Rotor-Gene Q still seems likely to prove a good alternative technology to yield more accurate measures, because wells are arranged in a ring, manual pipetting is difficult, multichannel pipettes cannot be used and costly robotics equipment are required to achieve high throughput.

In summary, we show that analysis strategy of qPCR TL data matters. In particular, in our data, well position correction yields power gains equivalent to an estimated 16% increase in sample size.

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