Effect of pre-analytic variables on the reproducibility of qPCR relative telomere length measurement

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Abstract

Telomeres, long nucleotide repeats and a protein complex at chromosome ends, shorten with each cell division and are susceptible to oxidative damage. Quantitative PCR (qPCR) is a widely-used technique to measure relative telomere length (RTL) in DNA samples but is challenging to optimize and significant lab-to-lab variability has been reported. In this study, we evaluated factors that may contribute to qPCR RTL measurement variability including DNA extraction methods, methods used for removing potential residual PCR inhibitors, sample storage conditions, and sample location in the PCR plate. Our results show that the DNA extraction and purification techniques, as well as sample storage conditions introduce significant variability in qPCR RTL results. We did not find significant differences in results based on sample location in the PCR plate or qPCR instrument used. These data suggest that lack of reproducibility in published association studies of RTL could be, in part, due to methodological inconsistencies. This study illustrates the importance of uniform sample handling, from DNA extraction through data generation and analysis, in using qPCR to determine RTL.

Introduction

Telomeres are comprised of $(TTAGGG)_n$ nucleotide repeats and a protein complex that protect chromosome ends [1]. They shorten with each cell division due to the inability of DNA polymerase to replicate the 3' end of DNA. Telomere length (TL) in blood or buccal cell DNA has been associated with cancer, heart disease, and several other illnesses [2–10].

Numerous TL measurement methods exist, each with advantages and limitations [11, 12]. PCR-based methods to measure TL are widely used because they require small amounts of DNA and are often less labor intensive than other methods. There are currently three reported methods to perform PCR analysis of TL: quantitative PCR (qPCR), monochrome multiplex qPCR (MMqPCR), and absolute telomere length qPCR (aTLqPCR). The first, qPCR, utilizes primers targeting the telomeric hexanucleotide repeats [13]. In the qPCR method, two separate qPCR reactions are performed and the resulting amount of telomere amplicons (T) are

compared to the amount of a single-copy gene amplicons (S) to generate the T/S ratio, resulting in a relative TL (RTL) rather than an absolute measure. The MMqPCR method performs both the T and the S reactions in the same well, thus reducing pipetting precision as a variable [14]. The aTLqPCR method adapted the original qPCR method to determine a base pair estimate of average TL by using a standard curve of synthesized telomeric repeat sequence oligonucleotide diluted to a known TL [15].

The original qPCR method, published in 2002, is the most widely used in large-scale studies, but has known variability within and between batches and lacks reference standards that are necessary to ensure consistency of results [11]. The correlation between qPCR RTL with other TL measurement methods, including terminal restriction fragment analysis (TRF) and flow cytometry with fluorescent *in situ* hybridization (flow FISH), varies with correlation coefficients (R^2) ranging from 0.1 to 0.99 [13, 16–20]. Many association studies using qPCR RTL measurement have not reported important details, such as DNA extraction methods, specific reagents and single copy loci used, as well as method of RTL value generation [12]. Others have shown that the DNA extraction method [21–26], tissue fixation method [27], and well position [28] are possible sources of variability in qPCR RTL measurement.

To address the factors contributing to qPCR RTL variability, we comprehensively evaluated the effects of DNA extraction method, PCR inhibitor removal methods, sample storage conditions, and sample location in the PCR plate.

Materials and methods

DNA extraction methods

Buffy coat specimens from 48 subjects, in the Research Donor Program at the Frederick National Laboratory for Cancer Research, were mixed thoroughly and split into three equal volume aliquots. Each homogenous aliquot was then extracted via QIAamp DNA Blood Midi Kit (Qiagen, Germantown, MD), QIAsymphony DNA Midi Kit (Qiagen), and ReliaPrep Large Volume HT gDNA Isolation System (Promega, Madison, WI). The QIAsymphony and ReliaPrep kits utilize magnetic bead/particle-based methods, while the QIAamp kit uses silica-membrane-based nucleic acid purification method. The DNA was quantified with Quant-iT PicoGreen dsDNA quantitation (Life Technologies, Grand Island, NY).

qPCR relative telomere length assay

DNA samples were transferred into 96-well plates and the concentration normalized to 1 ng/uL. We also randomly placed no template control (NTC) and internal quality control (QC) sample replicates, NA07057 (Coriell Cell Repositories, Camden, NJ), as calibrator samples. Four uL of DNA (4 ng) was then transferred, in triplicate, into quadrants 1, 2, and 3 of Light-Cycler-compatible 384-well plates (Roche, Indianapolis, IN) and a standard curve [6 concentrations of pooled reference DNA samples prepared by serial dilution (4 to .04096 ng/uL)] was added to quadrant 4 of each 384-well plate, all samples were dried down. This resulted in all experimental and control samples being assayed in triplicate on each 384-well plate for both T and S assays. All pipetting steps were performed using a Biomek FX (Beckman Coulter, Indianapolis, IN) liquid handler calibrated to perform transfers from 2–50 uL with a coefficient of variation (CV) of <5%.

Primers for the telomeric assay were *Telo_FP* [5′ -CGGTTT (GTTTGG) $_5$ GTT-3′] and *Telo_RP* [5′ -GGCTTG (CCTTAC) $_5$ CCT-3′] [29] and for the single-copy gene (36B4) assay were *36B4_FP* [5′ -CAGCAAGTGGGAAGGTGTAATCC-3′] and *36B4_RP* [5′ -CCCATTCTA TCATCAACGGGTACAA-3′] [13]. Primers (Integrated DNA Technologies, Coralville, IA) were manufactured LabReady (normalized to 100 uM in IDTE, pH 8.0 and HPLC Purified).

One μM assay mixes for each target were generated by combining 990 uL of 1X Tris-EDTA Buffer with 5 uL of forward oligo and 5 uL of reverse oligo.

PCR was performed using 5 uL reaction volumes consisting of: 2.5 uL of 2X Rotor-Gene SYBR Green PCR Master Mix (Qiagen), 2.0 uL of MBG Water, and 0.5 uL of 1 μM assay-specific mix. Thermal cycling was performed on a LightCycler 480 (Roche) where PCR conditions were (*i*) T (telomeric) PCR: 95°C hold for 5 minutes (min), denature at 98°C for 15 seconds, anneal at 54°C for 2 min, with fluorescence data collection, 35 cycles and (*ii)* S (single-copy gene, 36B4) PCR: 98°C hold for 5 min, denature at 98°C for 15 seconds, anneal at 58°C for 1 min, with fluorescence data collection, 43 cycles.

LightCycler software (Release 1.5.0) was used to generate Ct values, utilizing absolute quantification analysis with the second derivative maximum method and high sensitivity detection algorithm. Ct values or replicates were averaged, if they met a coefficient of variation (CV) threshold of less than 2%. The concentration (ng/uL) was interpolated from the plate-specific standard curve's exponential regression [Average Ct and log2 (Concentration)]. Any samples with 36B4 concentrations falling outside the range of the standard curve are dropped from further analysis as a T/S ratio cannot be accurately calculated. The telomere (T) concentration was divided by the 36B4 concentration (S) to yield a raw T/S ratio. The raw T/S ratio is divided by the average raw T/S ratio of the internal QC calibrator samples, within the same plate set, to yield a standardized T/S ratio that normalized results in reference to the same individual.

Evaluation of assay reproducibility

A single sample, the internal QC calibrator sample, was diluted to 1 ng/uL and then aliquoted into every well of a 96-well intermediate plate. This intermediate plate was used to aliquot this single sample, in triplicate, to twelve 384-well assay plates. Six assay plates were prepared with the Telomere assay and six with the 36B4 assay. Two plates for each assay were thermal cycled on three different LightCyclers.

DNA purification

After determining the baseline RTL, we applied three different DNA purification methods on 30 DNA samples from 10 subjects, extracted as described above (3 DNA samples/subject using different extraction techniques). The 30 DNA samples were mixed thoroughly and three 500 ng aliquots were created, which were purified using ethanol (EtOH) precipitation, MinElute (Qiagen), a silica-membrane-based purification, and AMPureXP (Beckman Coulter), a magnetic bead-based DNA capture method, creating 90 samples for qPCR analysis.

DNA storage temperature and concentration

We determined the baseline RTL of 50 different DNA samples and then subjected aliquots of these samples to different storage conditions for 6 months: 4°C at 25 ng/uL, 4°C at 1 ng/uL, -30°C at 25 ng/uL, and -30°C at 1 ng/uL.

Results

Well position and assay reproducibility

We first evaluated the reproducibility of the qPCR assay by measuring RTL on a single DNA sample aliquoted into a 96-well intermediate plate, then into 384-well plates for qPCR (S1 Dataset). The average amplification efficiency for the Telo assay was 96.66%, with a CV of 0.14% and for the 36B4 assay was 97.44%, with a CV of 0.25%. The average standardized T/S ratio for all RTL results (n = 576) was 1.00 and the CV was 2.20%. Three different Light Cyclers

were used to determine whether there was machine-to-machine variability. We ran 192 replicates on each LightCycler and found average standardized T/S ratios of 0.99, 1.00, and 1.00 with respective CVs of 2.58%, 1.79%, and 2.17%. Overall, the RTL results were reproducible and had little variability both within and across plates, run on various instruments.
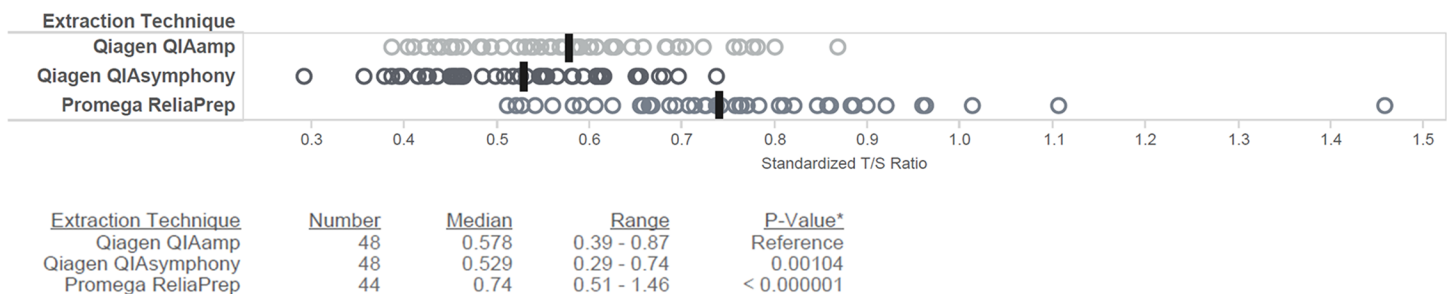
Evaluation of DNA extraction method

The median and range of RTL of buffy coat DNA varied by extraction method (Fig 1, S2 Dataset). The QIAamp RTL median T/S ratio was 0.58 (range 0.39–0.87). QIAsymphony median T/Swas 0.53 (range 0.29–0.74) and the ReliaPrep median was 0.74 (range 0.51–1.46). The median RTL differences between QIAamp and QIAsymphony or ReliaPrep, as determined by Wilcoxon signed rank test for paired samples, were statistically significant (p = 0.001, <0.001, respectively). For these samples, the CV for internal control replicates (n = 41) standardized T/S ratio was 5.13%. Correlation of RTL for the 48 matched subjects between extraction methods was modest (R^2 = 0.40, 0.54, and 0.54) as was Spearman's rank-order correlation (ρ = 0.53, 0.67, and 0.56) (Fig 2).

Evaluation of DNA purification techniques

The 30 DNA samples, three extraction methods from 10 matched subjects, underwent three different DNA purification methods (S3 Dataset). The CV for internal control replicates (n = 10) standardized T/S ratios was 8.01%. The magnitude of correlation was attenuated (R^2 = 0.68, ρ = 0.78) after the samples underwent additional purifications. When evaluating each inhibitor removal technique, the correlations of RTL of non-purified DNA to RTL after each purification type change slightly to, R^2 = 0.80, 0.76, and 0.59 (ρ = 0.88, 0.85, and 0.71) for MinElute, AMPureXP, and EtOH, respectively (Fig 3). The AMPureXP and MinElute techniques maintain a stronger correlation to results prior to purification than those of the EtOH technique, but each purification technique appears to affect the dynamic range differently, as indicated by the slope and intercept differences. The effect of DNA purification technique was independent of the DNA extraction technique for AMPureXP and MinElute. However, the EtOH purification technique for samples extracted via Promega ReliaPrep showed a stronger correlation post-purification (R^2 = 0.77, ρ = 0.83) than those samples from the same subject extracted via QIAamp (R^2 = 0.24, ρ = 0.47) and QIAsymphony (R^2 = 0.38, ρ = 0.59).

Evaluation of storage temperature and concentration

For this experiment, the CV for internal control replicates (n = 10) standardized T/S ratio was 6.03%. Samples stored at 25 ng/uL maintained strong correlations to the original results after 6

Extraction Technique	Number	Median	Range	P-Value*
Qiagen QIAamp	48	0.578	0.39 - 0.87	Reference
Qiagen QIAsymphony	48	0.529	0.29 - 0.74	0.00104
Promega ReliaPrep	44	0.74	0.51 - 1.46	< 0.000001

*Wilcoxon signed rank test for paired samples

Fig 1. Extraction techniques contribute to differences in dynamic range of relative telomere length. (Top) Dynamic range of RTL (standardized T/S ratio) by extraction technique in matched samples from the same subjects, median marked by black bar and (Bottom) count of samples, median standardized T/S ratio, range of standardized T/S ratio, and p-value for Wilcoxon signed rank test for paired samples by each extraction technique.

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months at both 4˚C ($R^2 = 0.86$) and -30˚C ($R^2 = 0.84$) storage temperatures. Samples normalized to 1 ng/uL were very weakly correlated to their original results when stored at 4˚C ($R^2 = 0.11$) and only moderately correlated when stored at -30˚C ($R^2 = 0.49$) (Fig 4, S4 Dataset).

Discussion

There is accumulating evidence that certain pre-analytic variables may be important in assuring reliability of the qPCR RTL measurements [12, 17, 21–23, 27]. Here, we assessed some of these variables including extraction technique, inhibitor removal technique, sample storage conditions, and assay plate location. Our results show that DNA extraction method, inhibitor removal techniques, and sample storage conditions significantly contribute to variability in RTL, while location of the sample on the assay plate has no or minor effect as previously observed [28].

Our data further illustrate the importance of using one DNA extraction method for an entire study. Alternatively, a DNA extraction method-specific calibrator could be used if
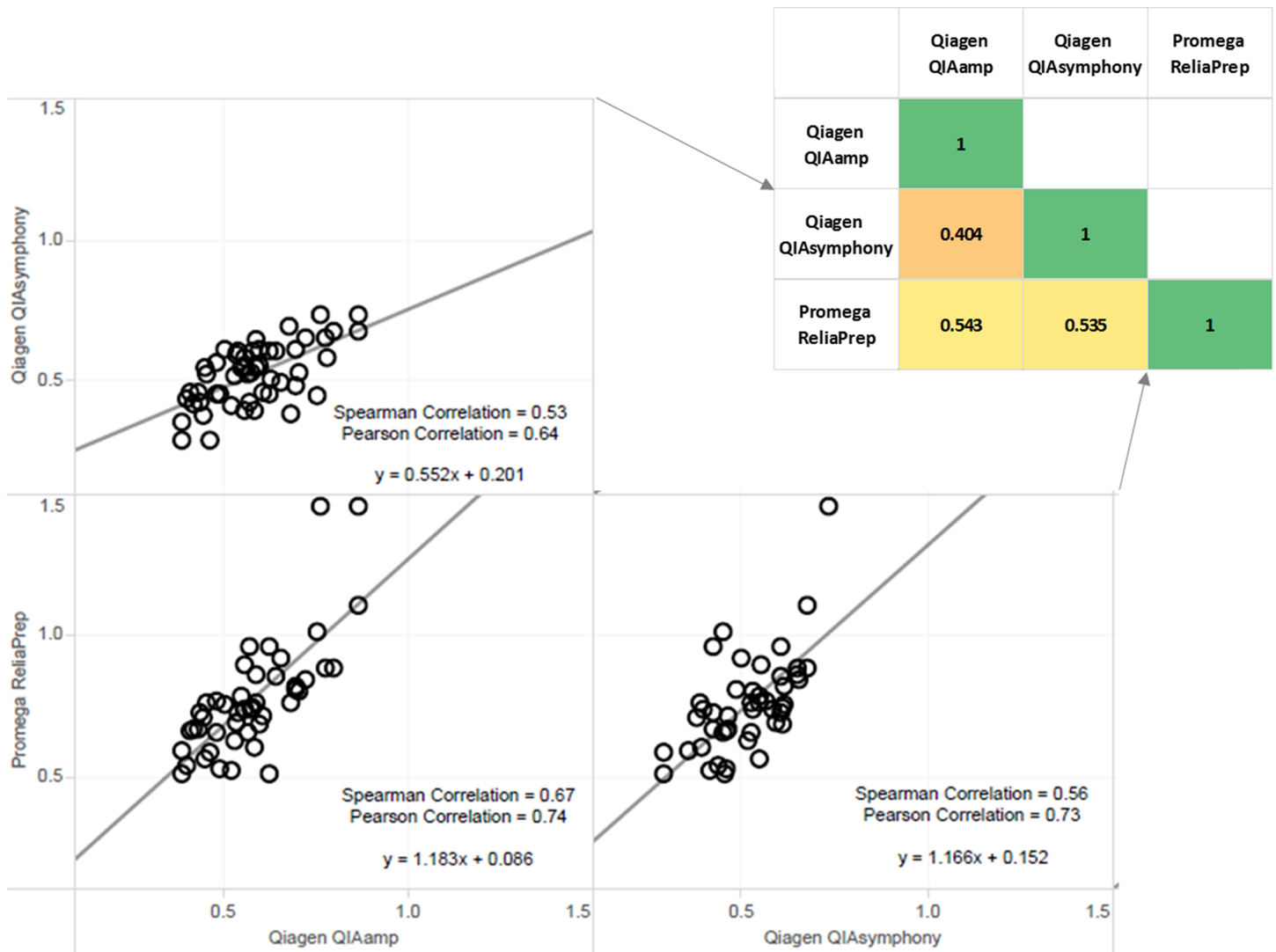

Fig 2. Correlation of relative telomere length (standardized T/S ratio) of matched subjects across extraction techniques and assay techniques. Inset heat map displays coefficient of determination (R^2) for each correlation.

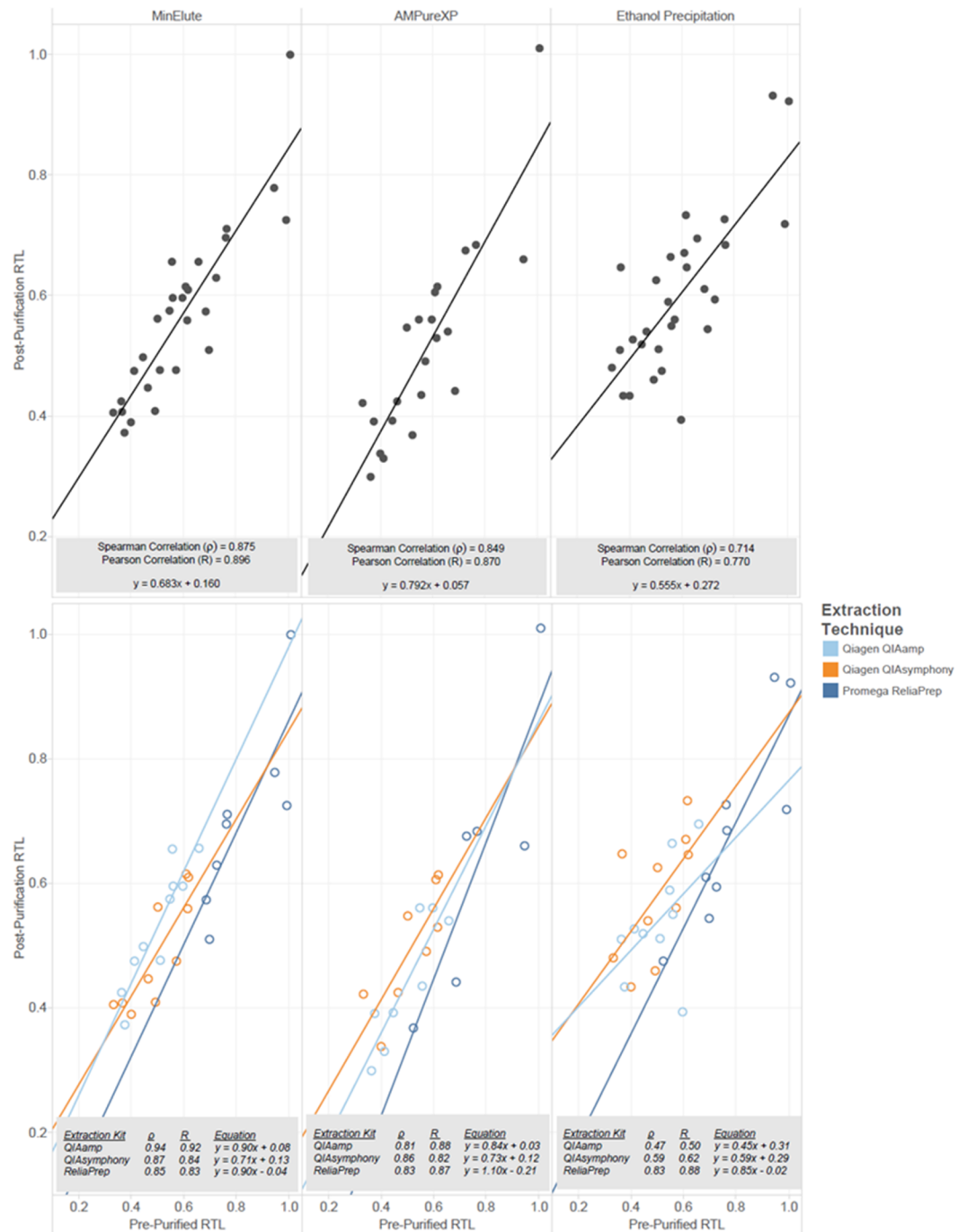https://doi.org/10.1371/journal.pone.0184098.g002

Fig 3. Correlation of relative telomere length (standardized T/S ratio) of matched samples pre- and post-purification. (Top) All samples by purification technique. (Bottom) By purification technique and extraction technique, shown by color, for 10 matched subjects extracted using three different techniques.

multiple extraction methods are planned, as recently described [26]. We confirmed that the use of different DNA extraction technique introduces variability in RTL measurement. Findings from previous studies evaluated DNA extraction techniques across different methodologies (either solid phase adsorption, magnetic bead adsorption, precipitation, or phenol-
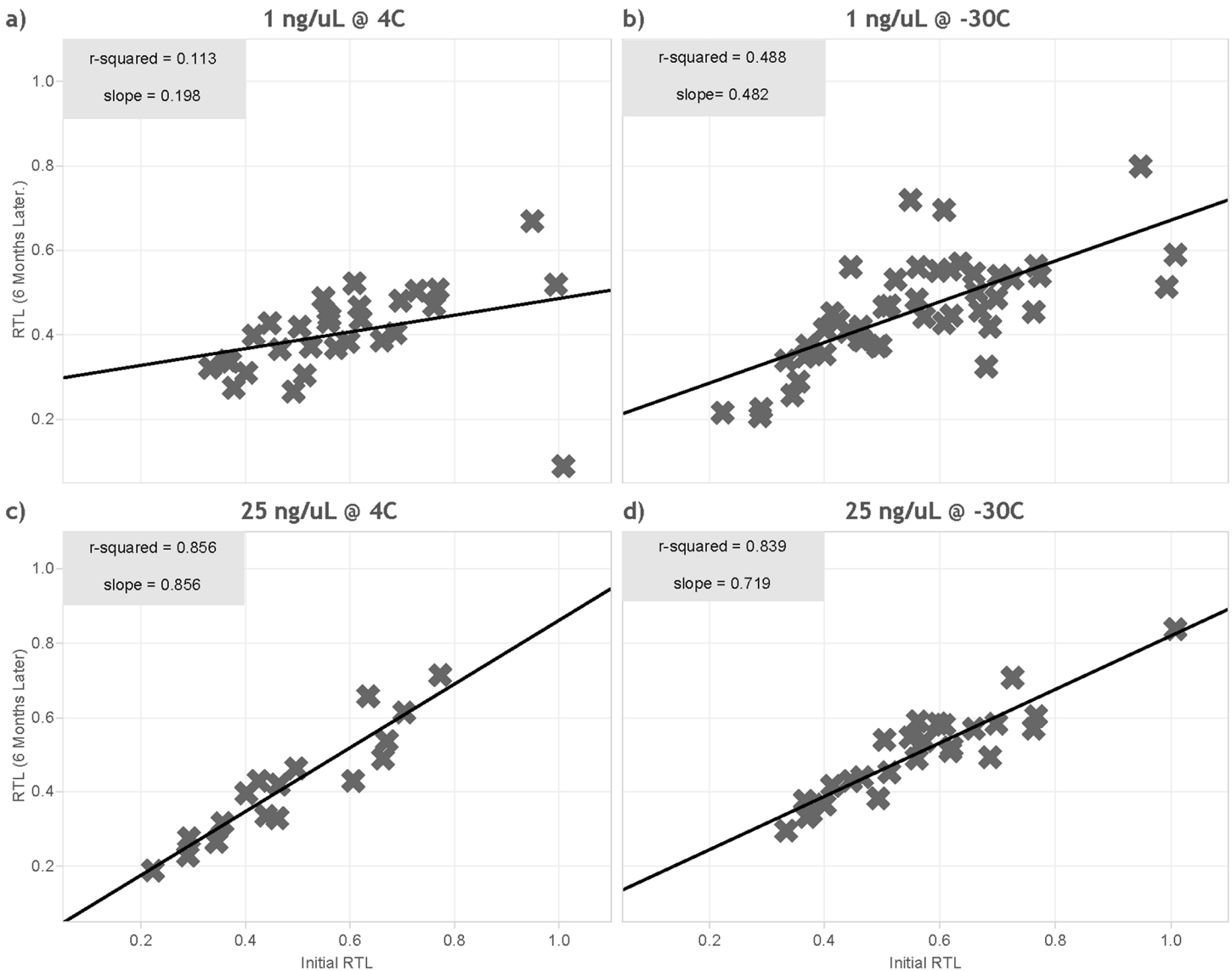
Fig 4. Correlation of relative telomere length (standardized T/S ratio) of same samples after 6 months at various concentrations and storage temperature conditions. (a) 1 ng/uL at 4°C, (b) 1 ng/uL at -30°C, (c) 25 ng/uL at 4°C, and (d) 25 ng/uL at -30°C.

chloroform) [21–23]. Here, we show that variability is also present between DNA extraction kits that use the same methodology. Specifically, we compared two different magnetic bead adsorption techniques (ReliaPrep and QIAsymphony) and showed that they not only had different dynamic ranges, but also had poor correlation of RTL in biological replicates.

Our data also demonstrated that variability can also be introduced in RTL by different inhibitor removal (purification) techniques, which are often necessary due to substances intrinsic to specific biological source materials or introduced during extraction or processing [30]. This variability further illustrates the importance of processing all samples within a study in the same manner.

The observed variability in RTL introduced by DNA sample storage temperature and concentration is not surprising as it is known that low concentration solutions of DNA are prone to DNA degradation or other loss over time [31]. However, the extent to which these factors

directly affect the telomeric repeat sequences in comparison to that of other regions of the genome, such as the single copy gene (36B4) is not known. This finding also suggests that internal controls utilized for assessment of reproducibility are subject to the same limitations.

In summary, this study shows that pre-analytic factors, including DNA extraction, purification, and storage introduce significant variability in qPCR RTL measurements. Our data show that studies with different pre-analytic methods may not be directly comparable. Therefore, we recommend that these factors be consistent within studies and that multiple replicates within and across studies are used. Researchers should strongly consider validating significant associations between qPCR RTL and disease in a different laboratory and, ideally, with a different measure of TL measurement.

Supporting information

S1 Dataset. Well position for assay reproducibility. Dataset contains 384-well plate descriptor (A, B, C), Well Position, Sample ID, LightCycler instrument number, and standardized T/S Ratio for the experiment regarding well position and assay reproducibility.
(XLSX)

S2 Dataset. DNA extraction method. Dataset contains blinded subject ID, extraction method utilized and standardized T/S Ratio for the experiment regarding DNA extraction method.
(XLSX)

S3 Dataset. DNA purification technique. Dataset contains blinded subject ID, extraction method utilized, purification technique utilized and standardized T/S ratio for the experiment regarding DNA purification technique and extraction method.
(XLSX)

S4 Dataset. Storage temperature and concentration. Dataset contains sample ID, standardized T/S ratio of initial analysis, standardized T/S ratio of analysis post-storage (6 months later), storage temperature (˚C) and concentration of DNA (ng/uL) prior to storage.
(XLSX)

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