Materials and Methods: Relative Telomere Length Assay

Relative telomere length determination by qPCR measures the ratio of telomere (T) signals, specific to the telomere hexamer repeat sequence TTAGGG, to autosomal single copy gene (S) signals. This ratio is normalized by control DNA samples to yield relative standardized T/S ratios proportional to average telomere length. In this technique reactions are performed independently, so a standard curve of pooled gDNA samples is utilized to assess the amount of each signal, while compensating for inter-plate variations in PCR efficiency. This telomere length measurement assay was adapted from the published method by Cawthon[1].

Quantitative PCR (qPCR)

For each reaction 4 ng of sample DNA, according to Quant-iT PicoGreen dsDNA quantitation (Life Technologies, Grand Island, NY), was transferred into LightCycler-compatible 384-well plates (Roche, Indianapolis, IN) and dried down. A standard curve [6 concentrations of pooled reference DNA samples prepared by serial dilution (4 to .04096 ng/uL)] and randomly located internal QC sample replicates (n=5), utilized as calibrator samples, to guide analysis and indicate overall quality of assay performance. Additionally, an NTC was added to random well locations to provide a unique fingerprint for each plate. All experimental and control samples were assayed in triplicate on each assay plate for both 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 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'][2] and for the single-copy gene (36B4) assay were *36B4_FP* [5'-CAGCAAGTGGGAAGGTGTAATCC-3'] and *36B4_RP* [5'-CCCATTCTATCATCAACGGGTACAA-3'][1]. Primers were manufactured LabReady, normalized to 100 uM in IDTE, pH 8.0 and HPLC Purified (Integrated DNA Technologies, Coralville, IA). 1 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, Germantown, MD), 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 min, denature at 98˚C for 15 s, 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 s, anneal at 58˚C for 1 min, with fluorescence data collection, 43 cycles.

The 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 of triplicates were averaged, if meeting a CV threshold of less than 2%, and the concentration (ng/uL) was interpolated from the plate-specific standard curve's exponential regression [Average Ct and log2(Concentration)]. Samples with 36B4 concentrations falling outside the range of the standard curve were dropped from further analysis. 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 to normalize results in reference to the same individual. Z-scores are calculated to adjust RTL in case differences in dynamic range are introduced by systematic differences between batches.

References

1. Cawthon, R.M. (2002) Telomere measurement by quantitative PCR. *Nucleic Acids Res.,* **30**, e47.
2. Callicott, R.J. and J.E. Womack (2006) Real-time PCR assay for measurement of mouse telomeres. *Comp. Med*., **56**, 17-22.