

2.4 Equipment

- QIAgility robotic pipettor (Qiagen)
- Rotordisk heat sealer Cat# 9018898 (Qiagen)
- RotorGene Q real-time PCR cycler (Qiagen)
- 5424R centrifuge (Eppendorf)
- Mini vortexer (VWR)
- Galaxy mini-centrifuge (VWR)
- 1500VA UPS (CyberPower)
- Nanodrop 2000 (Thermo Scientific)

3. EXPERIMENTAL PROCEDURES

3.1 Preparing Telomere and IFNB1 Standards

The cornerstone of the aTL assay is estimation of telomeric repeats and genome copy number for each sample via standard curves constructed using duplex oligomers.

Telomere Standard

The first telomere standard is constructed as a 0.15ng/ul dilution from the telomere duplex oligomer factor stock. This Standard A corresponds to containing 600pg DNA when 4uL is used in the qPCR assay. The number of telomere repeats in Standard A is calculated by:

$$\text{Weight of one molecule} = \text{Molar Mass} / \text{Avogadro's number} = \frac{51779.5 \text{ g/mol}}{6.022 \times 10^{23} \text{ mol}^{-1}} = 8.60 \times 10^{-20} \text{ g}.$$

$$\text{Molecules in Standard A} = \text{DNA per reaction} / \text{Weight of one molecule} = \frac{60.00 \times 10^{-11} \text{ g}}{8.60 \times 10^{-20} \text{ g}} = 6.98 \times 10^9.$$

Each oligomer is 84bp in length, thus

$$\text{Kb Telomeric DNA} = (\text{Molecules in Standard A} * 84) / 1000 = \frac{6.98 \times 10^9 * 84}{1000} = 5.86 \times 10^8 \text{ kb telomeric DNA}$$

Single Copy Gene Standard

The first single copy gene standard is constructed as 0.0005ng/ul dilution from the single-copy gene duplex oligomer factor stock. This Standard 1 corresponds to containing 2pg DNA when 4uL is used in the qPCR assay. The number of diploid copy numbers in Standard 1 is calculated by:

$$\text{Weight of one molecule} = \text{Molar Mass} / \text{Avogadro's number} = \frac{51161.2 \text{ g/mol}}{6.022 \times 10^{23} \text{ mol}^{-1}} = 8.496 \times 10^{-20} \text{ g}.$$

$$\text{Molecules in Standard 1} = \text{DNA per reaction} / \text{Weight of one molecule} = \frac{2.00 \times 10^{-12}}{8.496 \times 10^{-20}} = 2.35 \times 10^7.$$

There are two copies of this gene in a diploid genome, thus

$$\text{Diploid Copy Number} = (\text{Molecules in Standard 1}) / 2 = \frac{2.35 \times 10^7}{2} = 1.18 \times 10^7 \text{ diploid genomes}.$$

Oligomers used in construction of the above telomere and single copy gene standards are delivered as lyophilized pellet with variable yields following PAGE purification. The protocol below describes a general approach with equations provided as needed to account for variation in the delivered product. Steps 3.1.4 and 3.1.5 are performed using customized programs on the Qiagility robotic pipettor.

3.1.1 Resuspend lyophilized pellet containing oligomers for Telomere/IFNB1 standard in 1000uL 0.25x TE buffer. This *factory stock* can be stored in aliquots at -20°C for one year.

3.1.2 Make a *working stock* for each standard by combining 100uL *factory stock* with 900uL 0.25x TE buffer. This *working stock* can be stored in aliquots at -20°C for one year.

3.1.3 Measure OD260/280 of *working stock* on NanoDrop to determine DNA concentration and purity.

3.1.4a Use equation below to calculate volume *working stock* needed in a final 2mL volume of 0.25x TE for telomere Standard A. *another 1/10 dilution of the *working stock* may be necessary*

$$\text{Volume TELO Working Stock (uL)} = \frac{(1.50 \times 10^{-1} \text{ ng/uL})(2000 \text{ uL})}{\text{Concentration Working Stock } \left(\frac{\text{ng}}{\text{uL}}\right)}$$

3.1.4b Use equation below to calculate volume *working stock* needed in a final 2mL volume of 0.25x TE for the Single Copy Gene Standard 1. *another 1/10 dilution of the *working stock* may be necessary*

$$\text{Volume IFNB1 Working Stock (uL)} = \frac{(5.00 \times 10^{-4} \text{ ng/uL})(2000 \text{ uL})}{\text{Concentration Working Stock } \left(\frac{\text{ng}}{\text{uL}}\right)}$$

3.1.5 Perform 1/10 serial dilutions of Standard A and Standard 1 to construct remaining standards at 1500uL total volume. Use 0.25x TE buffer as the diluent. Aliquot in smaller quantities and freeze those not being used immediately. The ranges below are suitable for most applications.

Standard Range for Telomere Standards

Standard	ng/uL	pg DNA/reaction	Kb telo sequence
A	1.50e-01	600	5.86e+08
B	1.50e-02	60.0	5.86e+07
C	1.50e-03	6.0	5.86e+06
D	1.50e-04	0.6	5.86e+05
E	1.50e-05	0.06	5.86e+04
F	1.50e-06	0.006	5.86e+03
G	1.50e-07	0.0006	5.86e+02

Standard Range for IFNB1 Standards

Standard	ng/uL	pg DNA/reaction	Genome Copies
1	5.0e-04	2	1.18e+07
2	5.0e-05	0.2	1.18e+06
3	5.0e-06	0.02	1.18e+05
4	5.0e-07	0.002	1.18e+04
5	5.0e-08	0.0002	1.18e+03
6	5.0e-09	0.00002	1.18e+02
7	5.0e-10	0.000002	1.18e+01

3.2 PCR Set-Up

3.2.1 DNA Concentration

Calculate concentration of DNA samples using PicoGreen dsDNA binding assay (Thermo Scientific) using manufacturer's instructions. Dilute all samples to a uniform concentration at 1 ng/uL using 0.25x TE. Diluted samples can be stored at -20°C for up to 3 months.

3.2.2 Primer Working Stocks

Construct working stock (10uM) of TPF, TPR, IFNB1-F, and IFNB1-R primers. Make a working stock of 500uL total volume by adding 50uL factory stock to 450uL 0.25x TE buffer. Primer working stocks can be stored in aliquots at -20°C for one year before use. When running multiple assays several days in a row, store primers at 4°C to avoid freeze thaws. Primers stored at 4°C should be used within a month.

3.2.3 PCR Mastermix

Construct PCR mastermix appropriate for the number of samples on a given plate. Various compositions are detailed below. To accommodate the layout in Figure 1 one would need a 100x composition using the target primers for that plate and a 10x composition using the non-target primers (for control reactions only).

PCR Mastermix Construction

Reagent	1 rxn (uL)	5 rxn (uL)	10 rxn (uL)	50 rxn (uL)	100 rxn (uL)
SYBR Green PCR Mix	10	50	100	500	1000
10uM TPF/IFNB1-F	0.2	1.0	2.0	10	20
10uM TPR/IFNB1-R	0.2	1.0	2.0	10	20
UNG (1U/uL)	0.2	1.0	2.0	10	20
UltraPure PCR Water	3.4	17	34	170	340
Final Volume	14	70	140	700	1470

3.3 Plate Loading and PCR Cycling

3.3.1 Open the in-house Qiagility program template titled “Telomere_6standards_3controls” or “IFNB1_6standards_3controls” (*we do not use Telomere Standard G or IFNB1 Standard 7*) and load Rotor-disc 100 rotor with 100 well plates within the robotic pipette according to the layout detailed on the program. Once mastermix, standards, samples, controls, plasmid, and H₂O are in their appropriate positions, start the program. The 100-well disk is loaded as detailed in Figure 1. The contents of each well are described in Table 3.3.1. Plasmid DNA is added to standard wells to keep total ng DNA nearly equal for all wells except the blank. Store Qiagility Loading Blocks in the fridge when not in use.

Well Contents

	Standard Wells	Sample/Control Wells	Blank Well
Mastermix	14 uL	14 uL	14 uL
DNA (1.0 ng/uL)	-	6 uL	-
Standard A-G/1-7	4 uL	-	-
UltraPure H ₂ O	2 uL	-	6 uL
Total Volume	20 uL	20 uL	20 uL

3.3.2 Once the robot has completed loading of the rotor disk (~40 min), remove the entire 100-well ring loading block from the Qiagility instrument.

3.3.3 Apply heat-seal to the appropriate area of the block and tear along the perforated edge.

3.3.4 Load the 100-well loading block and seal onto the rotor-disk heat sealer, lift the sealing mechanism, and release once sealing is completed.

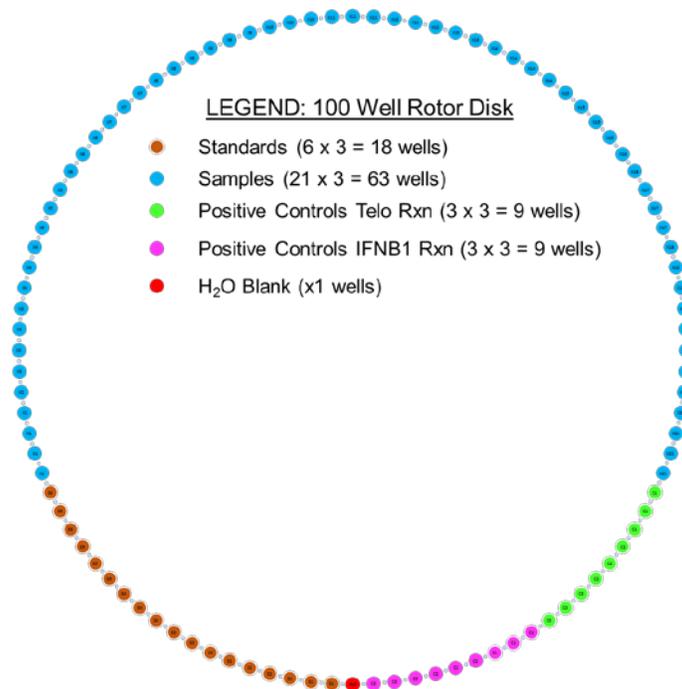
3.3.5 Remove the sealed 100-well disk from the loading block and load onto the RotorGene Q Rotor disc. Add locking ring and load into RotorGene Q for PCR cycling at the conditions below.

PCR Cycling

Hold at 50°C for 2 mins (<i>Activates UNG</i>)	
Hold at 95°C for 15 minutes	
95°C for 15 sec 55°C for 1 min (acquiring on Channel A) 72°C for 30 sec (acquiring on Channel B)	40 cycles
Melt curve generated ramping from 60°C to 99°C rising a degree each step after a 90 second pre-melt on the first step and 5 seconds each step afterwards	

3.3.6 A single telomere length assay consists of a telomere plate and an IFNB1 qPCR plate. Steps 3.3.1-3.3.5 must be done on ice for the telomere qPCR and repeated again on ice for the IFNB1 qPCR. The Qiagility loading blocks are kept in the fridge when not in use to maintain a cold temperature when being used on the Qiagility. As a rule of thumb, we run the telomere qPCR plate first. The Qiagility program is used to load the 100-well disk for the IFNB1 plate once the telomere plate has 30 minutes remaining on the thermocycler, using a freshly prepared IFNB1 mastermix. Samples are stored at 4°C for the duration between their use for the telomere plate and IFNB1 plate (~1.5 hours).

Figure 1: Sample Layout for 100-well plate



4. DATA ANALYSIS

4.1 Initial Processing within the Rotor-Gene Q Software

After each PCR run is completed, initial processing is conducted within the Rotor-Gene Q software. To begin this process click on the “Analysis” button on the top window. Navigate to the “Quantitation” tab, click “Cycling A Green” and click Ok. The window will now show normalized fluorescence profiles for each sample within the run. First ensure the “Dynamic Tube” option has been selected on the top toolbar. Next, determine C_q values using the “Auto-Find” threshold function with the default minimum and maximum values. Once C_q values have been calculated, inspect all standard replicates for outliers. Herein, inspect all standards, identifying those with standard deviation across replicate C_q values greater than 0.25. In instances where this occurs, inspect individual replicate values. If a given replicate C_q varies from the mean of two other replicates C_q values by more than 10%, it is marked as an outlier and dropped. Next recalculate the threshold using the Auto-Find threshold function following removal of outlier C_q values. Continue this process in an iterative fashion until no outliers remain. Note the final threshold determined using the Rotor-Gene Q software.

Repeat this process for all telomere and all single copy gene plates. Identify the most common threshold calculated by the Rotor-Gene Q software. This threshold will be used for all plates. Once identified, reanalyze all plates using this final threshold value. Once again inspect all standards for outliers using the process outlined above. Once all outlier standards have been dropped, note the efficiency value at this threshold for each plate. Turn all replicates back on and export the final C_q values to excel for calculation of T/S ratio values.

4.2 Aggregating Data in Excel

Organize the data in Excel in long format (one row per observation). The total number of rows (excluding headers) will be 200x the number of assays. When aggregating data in Excel it is helpful to add specific columns to assist with organizing the data. The specific columns are:

- **Origin:** a column at the far left of the worksheet that provides a unique identifier for each row
- **Amplicon:** a categorical variable to identify whether a given reaction was targeting telomeric content (0) or the single copy gene *IFNBI* (1).
- **Assay Number:** a column that specifies which assay the given reaction was from. Each assay consists of two plates, one with 100 telomere reactions and another with 100 *IFNBI* reactions
- **Sample Type:** a column that distinguishes between standard reactions (0), positive control reactions (1), and analytical sample reactions (2).

4.2 Controlling for Plate-to-Plate Variation

Cq values from standard reactions and positive control reactions are aggregated to account for plate-to-plate variation. First, the average Cq value across all T plates is calculated for each Standard A-F and for each control sample. For each standard and each control, a plate factor is calculated as $Factor_{i,j} = Cq_{\mu,i} - Cq_{i,j}$, where $Factor_{i,j}$ is the adjustment factor for the i^{th} sample on the j^{th} plate, $Cq_{\mu,i}$ is the mean Cq value for the i^{th} standard/control across all plates and $Cq_{i,j}$ is the mean value of the i^{th} standard/control on the j^{th} plate. This process is repeated across all standards and all controls to get the average factor for a given plate, and subsequently repeated across all T plates to generate factors for each plate. The same process is repeated across all S plates using Standards 1-6 and each control sample reaction targeting the single copy gene. Adjustment is performed independently for all T plates and all S plates. Cq values for analytical samples are adjusted using these plate factors prior to calculating T estimates and S estimates.

4.3 Calculating T Estimates and S Estimates from Cq Values

Estimates of telomeric content (T estimates) and genome copy number (S estimates) are calculated using the formula $T = E_T^{Cq_T}$ and $S = E_S^{Cq_S}$, where E_T and E_S are the average efficiency values across all T plates and S plates as determined by the Rotor-Gene Q software, and Cq_T and Cq_S are the Cq values for telomere and single copy gene reactions following adjustment for plate-to-plate variation.

4.4 Inspecting Replicate T Estimates and S Estimates

The coefficient of variation (CV) across replicate T estimates and S estimates is inspected for the purposes of identifying outliers. In instances, where the intra-assay CV for a given sample was greater than 15%, replicate values were inspected for outliers. If the T or S estimate for a given replicate deviated from the mean of the remaining two by more than 10% it was marked as an outlier and dropped. In these instances the sample-level T estimate and S estimate was calculated using the average of two replicates. If the CV across the two remaining replicates was still greater than 15% the entire sample was dropped and re-assayed a second time.

4.5 T/S Ratio Calculations

Final T/S ratio values are calculated using the formula $T/S = \left(\frac{T \text{ Estimate}}{S \text{ Estimate}}\right)^{-1}$.

REVISION HISTORY

DATE	AUTHOR	REVISION REASON
September 11, 2020	WJH	Updated to reflect new IFNB1 primer and oligomer duplex for standard curve
October 16, 2020	WJH	Use of plasmid pBR322 removed from protocol. Cycling profile updated. Addition of new section describing how to calculate aTL using LinRegPCR.
December 14, 2020	WJH	Increased each telomere standard by a power of ten such that suggested range is 5.86E+08 to 5.86E+02.
July 19, 2020	WJH	Removed section describing how to calculate aTL using LinRegPCR and replaced with section describing post-processing and calculation of T/S ratio estimates to reflect processes utilized for TRN DNA extraction experiment

References

1. Cawthon, R.M. (2002). Telomere measurement by quantitative PCR. *Nucleic acids research*, 30(10), e47-e47.
2. O'Callaghan, N.J., & Fenech, M. (2011). A quantitative PCR method for measuring absolute telomere length. *Biological procedures online*, 13(1), 3.