# TELOMERE LENGTH MEASUREMENT USING QPCR

## 1. PURPOSE & GENERAL OVERVIEW

This protocol describes the measurement of absolute telomere length (aTL) using qPCR, adapted by the Shalev Lab from previously published methods[1,2]. This approach approximates telomeric content per cell (T) using an 84bp oligomer standard composed of 14 repeats of the canonical telomere sequence in humans (TTAGGG). A second set of reactions amplified using the same DNA sample estimates the number of cells/genomes (S) using a second oligomer standard curve to quantitate a single-copy gene, interferon beta 1 (IFNB1). Dividing T by S reports the average total telomeric sequence per cell. A second division by 92 (23 chromosomal pairs with a telomere on each end) reports average length per telomere (in bp values).

A single qPCR assay can accommodate 22 samples (**Figure 1**), and is conducted across two 100-well plates. The first plate quantitates telomeric content, in triplicate, using telomere-specific primers and a standard curve. The second plate quantitates genome number, in triplicate, using single-copy gene primers and a standard curve. Five control samples are run on each plate to account for plate-to-plate variation. Reactions are prepared using a robotic pipettor (QIAgility, Qiagen) to ensure maximum pipetting accuracy, and real-time qPCR is performed with a unique rotary thermocycler (RotorGene Q, Qiagen), which reduces well position effects. Both instruments are connected to a uninterruptible power source (CP1500AVRLCD, CyberPower) to mitigate issues of electric power quality. This assay was developed and validated using a selection of samples with available Southern Blot Terminal Restriction Fragment (TRF) data, as shown in **Figure 2**.

## 2. MATERIALS/REAGENTS/EQUIPMENT

### 1.1 Primer sequences

| **Primer** | **Sequence** |
|---|---|
| TPF | 5'- CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT -3' |
| TPR | 5'- GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT -3' |
| IFNB1-F | 5’- TGG CAC AAC AGG TAG TAG GCG ACA C -3’ |
| IFNB1-R | 5’- GCA CAA CAG GAG AGC AAT TTG GAG GA -3’ |

Primers are purchased from IDT (www.idtdna.com) as premixed primer pairs (RxnReady® Primer Pool) with HPLC purification and 10uM concentration in IDTE Buffer pH 8.0.

### 1.2 Duplex oligonucleotide sequences

| **Oligomer** | **Sequence** |
|---|---|
| Telomere Standard | |
| Sense | 5’-CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA -3’ |

| | |
|---|---|
| Antisense | 5'- TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG-3' |
| IFNB1 Standard | |
| Sense | 5'- GCA CAA CAG GAG AGC AAT TTG GAG GAG ACA CTT GTT GGT CAT GTT GAC AAC ACG AAC AGT GTC GCC TAC TAC CTG TTG TGC CA -3' |
| Antisense | 5'- TGG CAC AAC AGG TAG TAG GCG ACA CTG TTC GTG TTG TCA ACA TGA CCA ACA AGT GTC TCC TCC AAA TTG CTC TCC TGT TGT GC -3' |

Oligomers are purchased from IDT as lyophilized pellet with PAGE purification. Purchasing item labeled "100nmole Duplex" typically results in a yield of 1.5-4.5nmol lyophilized oligomer upon delivery.

### 1.3 Reagents and supplies

| **Material** | **Vendor** | **Cat#** |
|---|---|---|
| SYBR Green PCR Kit | Qiagen | 204145 |
| Uracil-DNA glycosylase (UNG) | Thermo Scientific | FEREN0362 |
| TE Buffer (20x) | Thermo Scientific | T11493 |
| 100 well rotor disk plate | Qiagen | 981311 |
| Rotor-disc 100 Rotor | Qiagen | 9081195 |
| Rotor-disc 100 locking ring | Qiagen | 9018896 |
| 50uL Conductive Filtered Tips (Qiagility) | Qiagen | 990512 |
| 200uL Conductive Filtered Tips (Qiagility) | Qiagen | 990522 |
| Rotor disk heat sealing film | Qiagen | 981601 |
| 200uL PCR tubes | Qiagen | 981005 |
| 5mL diluent tube | Qiagen | 990552 |
| 15mL conical tube | VWR | 89126-798 |
| 50mL conical tube | VWR | 89126-802 |
| 2mL cryovial | VWR | 10018-754 |
| 1.5mL microcentrifuge tube | VWR | 20170-038 |
| 2.0mL microcentrifuge tube | Fisher | 26B1271 |
| Electronic serological pipette | Eppendorf | 4430000018 |
| 10mL serological pipette tips | Eppendorf | 0030127722 |
| 25mL serological pipette tips | Eppendorf | 0030127730 |
| UltraPure Sterile $H_2O$ | VWR | RLMB-010-0100 |
| Pipettes (0.2-2uL, 2-20uL, 20-200uL, & 100-1000uL) | Thermo Scientific | 4700880 |
| 10uL filter tips, sterile | VWR | 89174-520 |
| 20uL filter tips, sterile | VWR | 89174-524 |
| 200uL filter tips, sterile | VWR | 89174-526 |
| 1000uL filter tips, sterile | VWR | 89174-530 |

### 1.4 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 0.10ng/ul dilution from the telomere duplex oligomer factor stock. This Standard A corresponds to containing 600pg DNA when 6uL is used in the qPCR assay. The number of telomere repeats in Standard A is calculated by:

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

Molecules in Standard A = DNA per reaction / Weight of one molecule = $\frac{6.00\ x\ 10^{-10} g}{8.60\ x\ 10^{-20} g} = 6.98\ x\ 10^{9}.$

Each oligomer is 84bp in length, thus

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

Single Copy Gene Standard

The first single copy gene standard is constructed as 0.00033ng/ul dilution from the single-copy gene duplex oligomer factor stock. This Standard 1 corresponds to containing 2pg DNA when 6uL is used in the qPCR assay. The number of diploid copy numbers in Standard 1 is calculated by:

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

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

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

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

**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*

$$Volume\ TELO\ Working\ Stock\ (uL) = \frac{(1.00\ x10^{-1}\ ng/uL\ )(2000\ uL)}{Concentration\ Working\ Stock\ (\frac{ng}{uL})}$$

**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. *several 1/10 dilutions of the *working stock* may be necessary*

$$Volume\ IFNB1\ Working\ Stock\ (uL) = \frac{(3.33\ x10^{-4}\ ng/uL\ )(2000\ uL)}{Concentration\ Working\ Stock\ (\frac{ng}{uL})}$$

**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 | 1.00e-01 | 600 | 5.86e+08 |
| B | 1.00e-02 | 60.0 | 5.86e+07 |
| C | 1.00e-03 | 6.0 | 5.86e+06 |
| D | 1.00e-04 | 0.6 | 5.86e+05 |
| E | 1.00e-05 | 0.06 | 5.86e+04 |
| F | 1.00e-06 | 0.006 | 5.86e+03 |

**Standard Range for IFNB1 Standards**

| Standard | ng/uL | pg DNA/reaction | Genome Copies |
|---|---|---|---|
| 1 | 3.33e-04 | 2 | 1.18e+07 |
| 2 | 3.33e-05 | 0.2 | 1.18e+06 |
| 3 | 3.33e-06 | 0.02 | 1.18e+05 |
| 4 | 3.33e-07 | 0.002 | 1.18e+04 |
| 5 | 3.33e-08 | 0.0002 | 1.18e+03 |
| 6 | 3.33e-09 | 0.00002 | 1.18e+02 |

## 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

Aliquot combined primer solutions into 50uL aliquots for use in 110 rxn mastermix shown in Table 3.2.3. Primer aliquots can be stored in aliquots at -20°C for one year before use. After aliquoted in this manner, the 1000uL primers pair ordered from IDT is typically sufficient for 18 assays. If more than this amount is needed for a given patch of assays a custom order can be submitted to IDTquotes@idtdna.com.

### 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 110x composition using the target primers for that plate.

**Table 3.2.3 PCR Mastermix Construction**

| Reagent | 1 rxn (uL) | 10 rxn (uL) | 50 rxn (uL) | 100 rxn (uL) | 110 rxn (uL) |
|---|---|---|---|---|---|
| SYBR Green PCR Mix | 10 | 100 | 500 | 1000 | 1100 |
| 10uM Telo/IFNB1 Primer Pair | 0.4 | 4.0 | 20 | 40 | 44 |
| UNG (1U/uL) | 0.2 | 2.0 | 10 | 20 | 22 |
| UltraPure PCR Water | 3.4 | 34 | 170 | 340 | 374 |
| Final Volume | 14 | 140 | 700 | 1400 | 1540 |

## 3.3 Plate Loading and PCR Cycling

**3.3.1** Open the in-house Qiagility program template located at "Desktop/Waylon/Unique Templates/Telomere Assay Both Plates_5controls_6standards_22samples_noStandardH2O" 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, and $H_2O$ 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.

**Table 3.3.1 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 | 6 uL | - | - |
| UltraPure $H_20$ | - | - | 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 (*Activates UNG*) | |
| Hold at 95°C for 15 minutes | |
| 95°C for 15 sec<br>55°C for 1 min (acquiring on Channel A) | 40 cycles |
| Melt curve generated ramping from 60°C to 95°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. 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: Layout for 100-well plate**

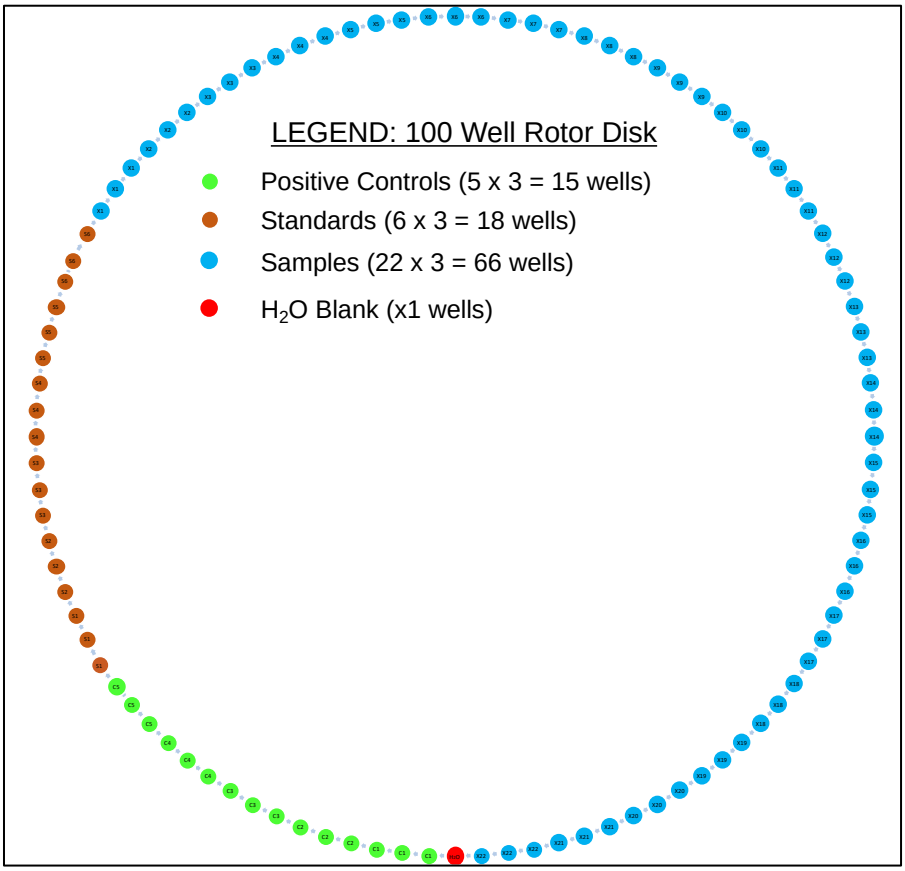
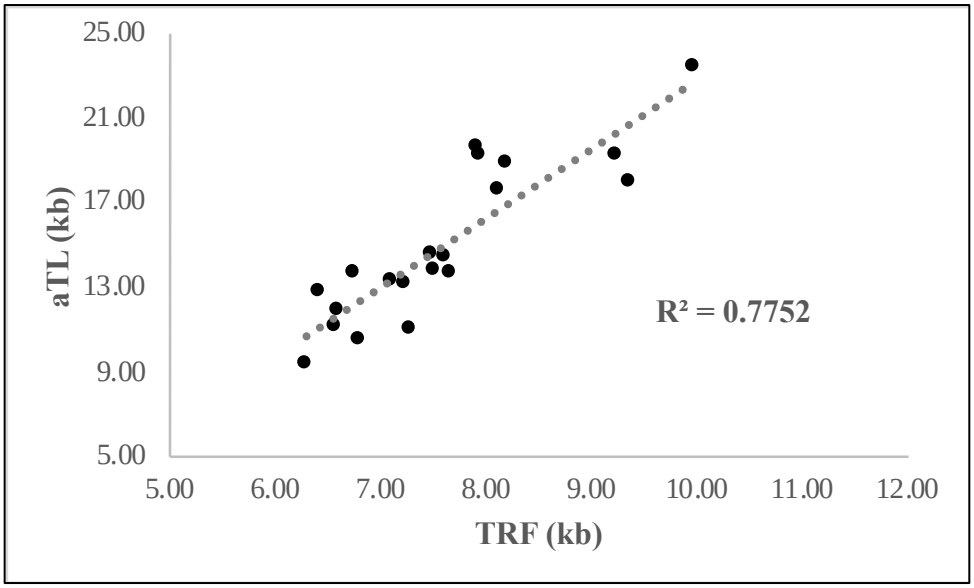



**Figure 2: Scatter Plot of aTL and TRF Measurements**



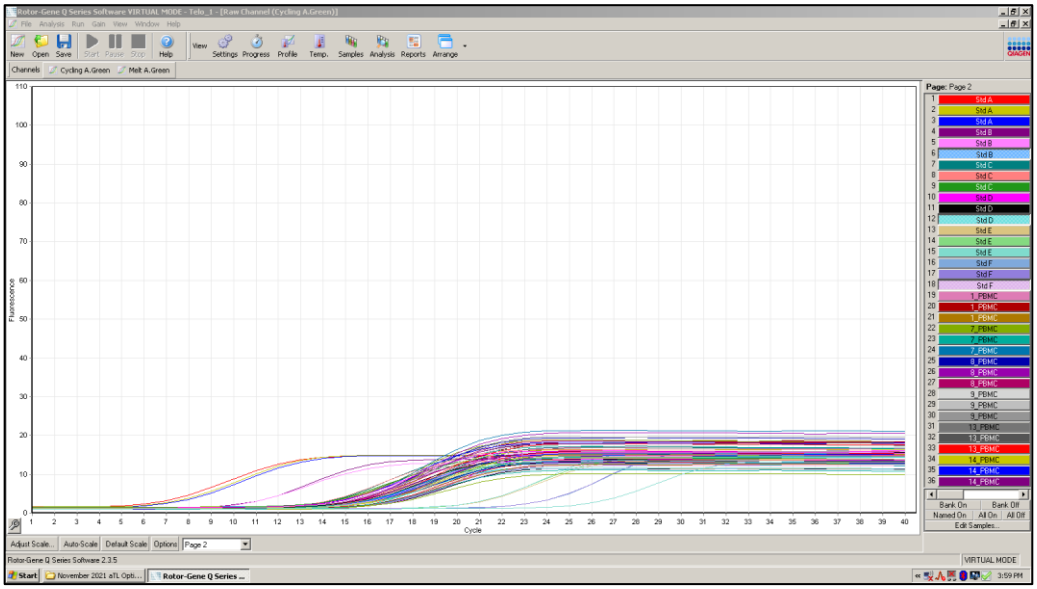
## 4. DATA ANALYSIS USING ROTORGENE INSTRUMENT

Primary data analysis and calculation of aTL values is conducted using estimates of telomeric content (in kilobases) and genome copy number determined by the RotorGene instrument software using the oligomer standard curve. The steps described here can be initiated once a given qPCR plate has been completed.

### 4.1 Determining Fluorescence Threshold and Inspecting Standard Curve

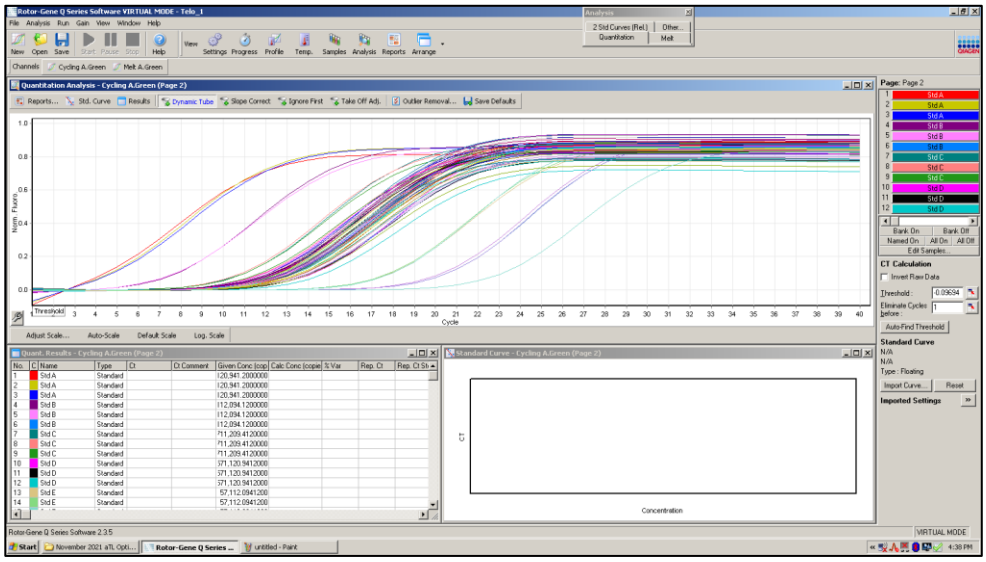
**4.1.1** Once the qPCR run has been completed a curve of the raw fluorescence will be illustrated for each sample as shown in **Figure 4.1**.

**Figure 4.1: Completed RotorGene Run**



**4.1.2** To determine estimated kb telomeric DNA and genome copy number click the “Analysis” tab. A pop-up submenu will appear. Navigate to the “Quantitation” subtab, click “Cycling A Green (page 2)” and click “Show”. The view will change to display three different windows as illustrated in **Figure 4.2** below.

**Figure 4.2: Default Analysis Screen**



**4.1.3** Click the "Dynamic Tube" setting if this has not already been selected. This setting slightly alters how the software determines background fluorescence values for each sample and in practice tends to result in decreased variation between Ct values between replicates of the same sample. Next navigate to the threshold box on the middle right of the window. This box allows one to manually set a threshold to determine Ct values for each. Alternatively, one can use the "Auto-Find Threshold" feature to have the software determine the threshold at which the $R^2$ of the standard curve is maximized. In practice however, this auto-determined threshold might vary between different runs. Therefore to ensure the same threshold is utilized across all plates we set the threshold manually and have found a value of 0.10 tends to produce reproducible results and be near the values selected by the Auto-Find function.

**4.1.4** Once the threshold has been set a standard curve will be generated in the bottom right window. Along with this each reaction will have Ct values corresponding to the cycle at which they cross this threshold and an estimated kb telomeric DNA/genome copy number in accordance with how their Ct value aligns with that of the standard curve (**Figure 4.3**). In some cases however one of the standards will have a clear outlier replicate as is shown for Standard B in **Figure 4.3**. Since the estimations for kb telomeric DNA and genome copy numbers are based on the alignment of the standards with this curve, the outlier standard replicate can negatively impact the resulting estimations. In this case it is advisable to drop this replicate before exporting the data. To do so simply navigate to the sample bank at the top left and click off the outlier replicate. Repeat this process with any other outliers, **defined as instances where both the following conditions are met 1) the standard deviation across replicate Ct values is greater than 0.10 and 2) the absolute deviation of the potential outlier Ct value to the closest replicate Ct value is more than twice the deviation between the remaining two replicates.** In this instance dropping the outlier replicate from Standard B decreases the estimated efficiency from 1.13 to 1.04 and increases the $R^2$ from 0.92867 to 0.99795 (**Figure 4.4**). Record the final $R^2$ and efficiency for subsequent reporting. As a rule of thumb $R^2$ values should be greater than 0.99 and efficiency values should range between 0.90 and 1.10.

**Figure 4.3: Standard Curve with Outlier Replicate**

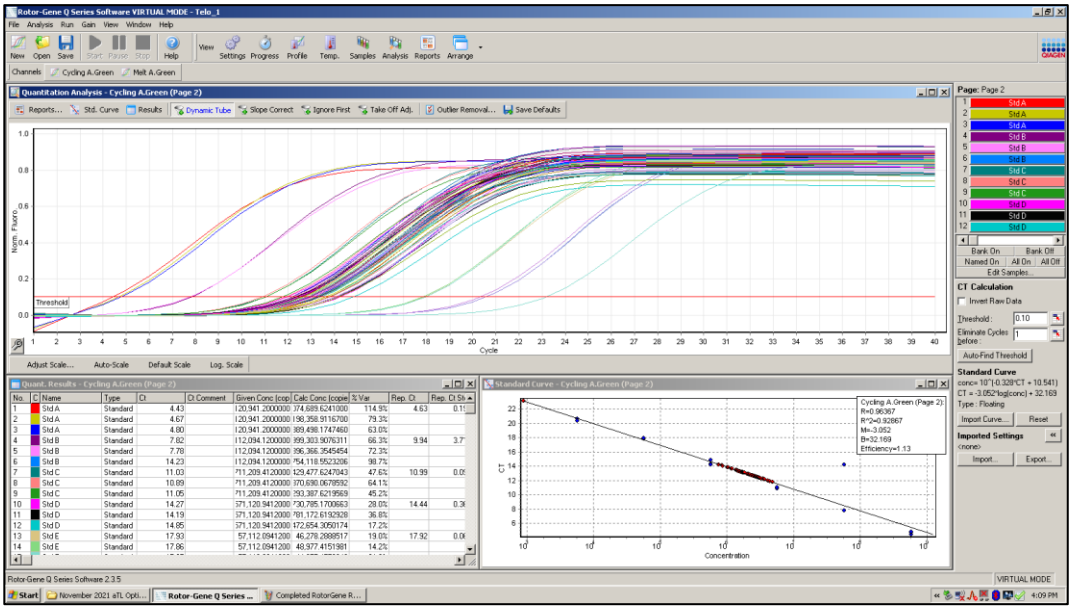
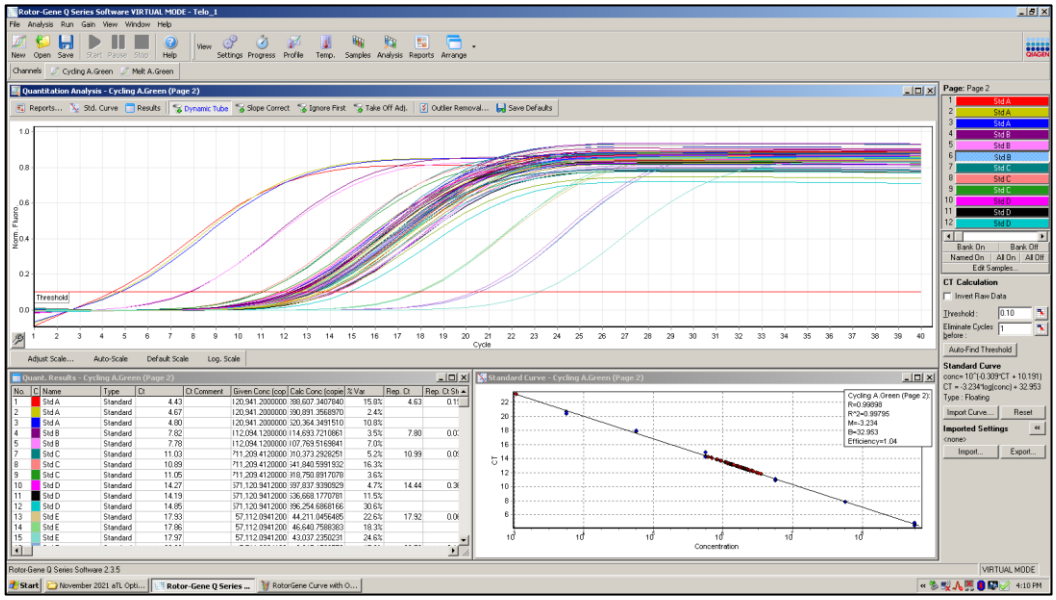


**Figure 4.4: Standard Curve without Outlier Replicate**



## 4.2 Exporting RotorGene Data

Once the standard curve has been finalized and assay quality metrics have been recorded the data can be exported for aTL calculations. To export the data simply right click on the analysis table at the bottom right and select “Export to Excel”. Give the file an informative name. The strategy in our lab is to name the file in accordance with the amplicon (i.e., Telo or IFNB1) followed by a suffix which indicates assay number (e.g., Telo_1, IFNB1_15, etc.). The exported data will provide the well ID, sample ID, sample type, Ct values, and estimated concentration for each reaction (other than outlier replicates of standards that were dropped) as indicated in **Figure 4.5**.

**Figure 4.5: RotorGene Analysis Export**

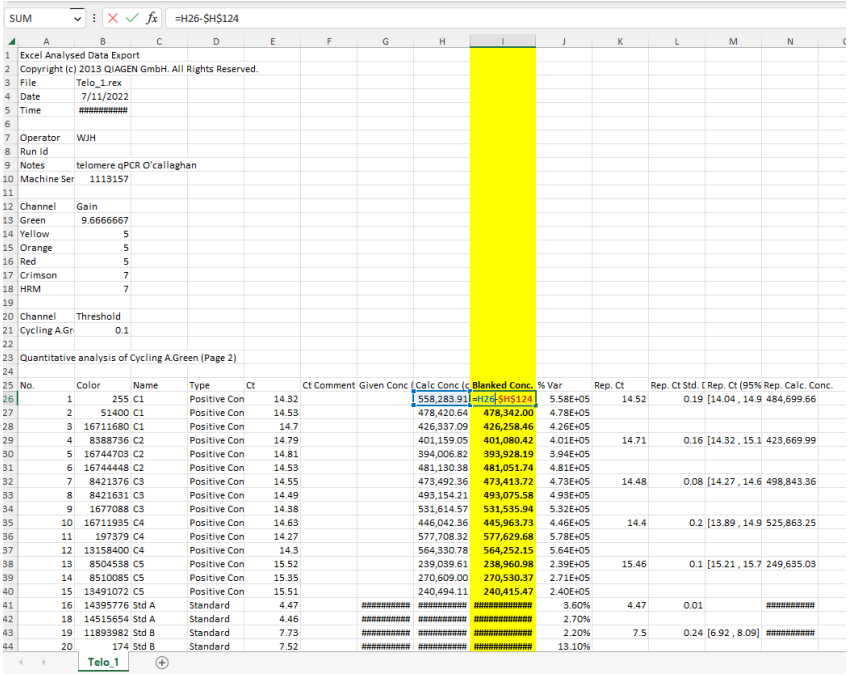
| | A | B | C | D | E | F | G | H | I | J | K | L | M |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Excel Analysed Data Export | | | | | | | | | | | | |
| 2 | Copyright (c) 2013 QIAGEN GmbH. All Rights Reserved. | | | | | | | | | | | | |
| 3 | File | Telo_1.rex | | | | | | | | | | | |
| 4 | Date | 5/20/21 | | | | | | | | | | | |
| 5 | Time | 1:04:07 PM | | | | | | | | | | | |
| 6 | | | | | | | | | | | | | |
| 7 | Operator | WJH | | | | | | | | | | | |
| 8 | Run Id | | | | | | | | | | | | |
| 9 | Notes | telomere qPCR O'callaghan | | | | | | | | | | | |
| 10 | Machine Ser | 1113157 | | | | | | | | | | | |
| 11 | | | | | | | | | | | | | |
| 12 | Channel | Gain | | | | | | | | | | | |
| 13 | Green | 9.66666667 | | | | | | | | | | | |
| 14 | Yellow | 5 | | | | | | | | | | | |
| 15 | Orange | 5 | | | | | | | | | | | |
| 16 | Red | 5 | | | | | | | | | | | |
| 17 | Crimson | 7 | | | | | | | | | | | |
| 18 | HRM | 7 | | | | | | | | | | | |
| 19 | | | | | | | | | | | | | |
| 20 | Channel | Threshold | | | | | | | | | | | |
| 21 | Cycling A.Gre | 0.1 | | | | | | | | | | | |
| 22 | Melt A.Greei | 1.14085 | | | | | | | | | | | |
| 23 | | | | | | | | | | | | | |
| 24 | Quantitative analysis of Cycling A.Green (Page 2) | | | | | | | | | | | | |
| 25 | | | | | | | | | | | | | |
| 26 | No. | Color | Name | Type | Ct | Ct Comment | Given Conc ( | Calc Conc (cc | % Var | Rep. Ct | Rep. Ct Std. I | Rep. Ct (95% | Rep. Calc. Conc |
| 27 | 1 | 255 | Std A | Standard | 4.43 | | ########## | ########## | 11.50% | 4.63 | 0.19 | [4.17 , 5.10] | ########## |
| 28 | 2 | 51400 | Std A | Standard | 4.67 | | ########## | ########## | 5.90% | | | | |
| 29 | 3 | 16711680 | Std A | Standard | 4.8 | | ########## | ########## | 13.90% | | | | |
| 30 | 4 | 8388736 | Std B | Standard | 7.82 | | ########## | ########## | 1.20% | 7.8 | 0.03 | | ########## |
| 31 | 5 | 16744703 | Std B | Standard | 7.78 | | ########## | ########## | 4.60% | | | | |
| 32 | 7 | 8421376 | Std C | Standard | 11.03 | | ########## | ########## | 4.30% | 10.99 | 0.09 | [10.78 , 11.2 | ########## |
| 33 | 8 | 8421631 | Std C | Standard | 10.89 | | ########## | ########## | 15.20% | | | | |
| 34 | 9 | 1677088 | Std C | Standard | 11.05 | | ########## | ########## | 2.70% | | | | |
| 35 | 10 | 16711935 | Std D | Standard | 14.27 | | 571,120.94 | 600,874.40 | 5.20% | 14.23 | 0.06 | | 619,962.92 |
| 36 | 11 | 197379 | Std D | Standard | 14.19 | | 571,120.94 | 639,657.84 | 12.00% | | | | |
| 37 | 13 | 8504538 | Std E | Standard | 17.93 | | 57,112.09 | 45,142.57 | 21.00% | 17.92 | 0.06 | [17.78 , 18.0 | 45,542.03 |

### 4.3 Inspecting No-Template Control

Well 100 of each qPCR run hosts a no-template control (NTC) reaction containing sterile $H_20$ and the PCR mastermix (see **Table 3.3.1**). In ideal circumstances, there should be no amplification in the NTC well. In instances when the reaction does show amplification, it implies that the mastermix was contaminated and/or primer dimers were formed. Fortunately, if this well does amplify, it commonly does so well below the lowest concentrated standard, resulting in estimated values that are significantly lower than the values for the analytical samples, thereby minimizing experimental error. For example, the NTC in Figure 4.5 crosses the detection threshold between cycles 23 and 24, while the lowest standard crosses the same threshold between cycles 20 and 21. In practice, when the NTC well does amplify, it's estimated value tends to be less than 2% of the average estimates for analytical samples on IFNB1 plates and less than 0.50% of the average estimates for analytical samples on telomere plates. **In instances when the NTC well amplifies above the lowest concentrated standard, or has an estimated genome copy number and/or kb telomeric DNA with value greater than 5% of the average estimates for the analytical samples on that same plate, then the whole plate should be discarded and rerun**.

In instances when the NTC well does amplify, but has an estimated value less than 5% of the analytical samples, it will be mitigated by 'blanking' the remaining samples on that plate prior to data aggregation. Blanking the remaining samples consists of subtracting the estimated kb telomeric DNA and/or estimated genome copy number for the NTC well from the values estimated for the analytical samples. This is accomplished by inserting an additional column into the exported RotorGene datasheet titled "Blanked Conc" whose values are calculated using the equation $Blanked\ Conc_i = Calc\ Conc_i - Calc\ Conc_{NTC}$, where *i* is a given analytical sample replicate. See **Figure 4.6** for an example of this setup where the new column is bolded and highlighted for emphasis and the calculation is provided in the formula bar at top (*Note: the Calc Conc for the NTC well is found in cell H124*).

**Figure 4.6: RotorGene Analysis Export with NTC Blanking**



SUM | =H26-$H$124

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Excel Analysed Data Export | | | | | | | | | | | | | |
| 2 | Copyright (c) 2013 QIAGEN GmbH. All Rights Reserved. | | | | | | | | | | | | | |
| 3 | File | Telo_1.rex | | | | | | | | | | | | |
| 4 | Date | 7/11/2022 | | | | | | | | | | | | |
| 5 | Time | ########## | | | | | | | | | | | | |
| 6 | | | | | | | | | | | | | | |
| 7 | Operator | WJH | | | | | | | | | | | | |
| 8 | Run Id | | | | | | | | | | | | | |
| 9 | Notes | telomere qPCR O'callaghan | | | | | | | | | | | | |
| 10 | Machine Ser | 1113157 | | | | | | | | | | | | |
| 11 | | | | | | | | | | | | | | |
| 12 | Channel | Gain | | | | | | | | | | | | |
| 13 | Green | 9.6666667 | | | | | | | | | | | | |
| 14 | Yellow | 5 | | | | | | | | | | | | |
| 15 | Orange | 5 | | | | | | | | | | | | |
| 16 | Red | 5 | | | | | | | | | | | | |
| 17 | Crimson | 7 | | | | | | | | | | | | |
| 18 | HRM | 7 | | | | | | | | | | | | |
| 19 | | | | | | | | | | | | | | |
| 20 | Channel | Threshold | | | | | | | | | | | | |
| 21 | Cycling A.Gr | 0.1 | | | | | | | | | | | | |
| 22 | | | | | | | | | | | | | | |
| 23 | Quantitative analysis of Cycling A.Green (Page 2) | | | | | | | | | | | | | |
| 24 | | | | | | | | | | | | | | |
| 25 | No. | Color | Name | Type | Ct | Ct Comment | Given Conc | Calc Conc | Calc Conc (c | **Blanked Conc.** | % Var | Rep. Ct | Rep. Ct Std. D | Rep. Ct (95% | Rep. Calc. Conc. |
| 26 | 1 | 255 | C1 | Positive Con | 14.32 | | | 558,283.91 | | =H26-$H$124 | 5.58E+05 | 14.52 | 0.19 | [14.04 , 14.9 | 484,699.66 |
| 27 | 2 | 51400 | C1 | Positive Con | 14.53 | | | 478,420.64 | | 478,342.00 | 4.78E+05 | | | | |
| 28 | 3 | 16711680 | C1 | Positive Con | 14.7 | | | 426,337.09 | | 426,258.46 | 4.26E+05 | | | | |
| 29 | 4 | 8388736 | C2 | Positive Con | 14.79 | | | 401,159.05 | | 401,080.42 | 4.01E+05 | 14.71 | 0.16 | [14.32 , 15.1 | 423,669.99 |
| 30 | 5 | 16744703 | C2 | Positive Con | 14.81 | | | 394,006.82 | | 393,928.19 | 3.94E+05 | | | | |
| 31 | 6 | 16744448 | C2 | Positive Con | 14.53 | | | 481,130.38 | | 481,051.74 | 4.81E+05 | | | | |
| 32 | 7 | 8421376 | C3 | Positive Con | 14.55 | | | 473,492.36 | | 473,413.72 | 4.73E+05 | 14.48 | 0.08 | [14.27 , 14.6 | 498,843.36 |
| 33 | 8 | 8421631 | C3 | Positive Con | 14.49 | | | 493,154.21 | | 493,075.58 | 4.93E+05 | | | | |
| 34 | 9 | 1677088 | C3 | Positive Con | 14.38 | | | 531,614.57 | | 531,535.94 | 5.32E+05 | | | | |
| 35 | 10 | 16711935 | C4 | Positive Con | 14.63 | | | 446,042.36 | | 445,963.73 | 4.46E+05 | 14.4 | 0.2 | [13.89 , 14.9 | 525,863.25 |
| 36 | 11 | 197379 | C4 | Positive Con | 14.27 | | | 577,708.32 | | 577,629.68 | 5.78E+05 | | | | |
| 37 | 12 | 13158400 | C4 | Positive Con | 14.3 | | | 564,330.78 | | 564,252.15 | 5.64E+05 | | | | |
| 38 | 13 | 8504538 | C5 | Positive Con | 15.52 | | | 239,039.61 | | 238,960.98 | 2.39E+05 | 15.46 | 0.1 | [15.21 , 15.7 | 249,635.03 |
| 39 | 14 | 8510085 | C5 | Positive Con | 15.35 | | | 270,609.00 | | 270,530.37 | 2.71E+05 | | | | |
| 40 | 15 | 13491072 | C5 | Positive Con | 15.51 | | | 240,494.11 | | 240,415.47 | 2.40E+05 | | | | |
| 41 | 16 | 14395776 | Std A | Standard | 4.47 | | ########## | ########## | | ########## | 3.60% | 4.47 | 0.01 | | ########## |
| 42 | 18 | 14515654 | Std A | Standard | 4.46 | | ########## | ########## | | ########## | 2.70% | | | | |
| 43 | 19 | 11893982 | Std B | Standard | 7.73 | | ########## | ########## | | ########## | 2.20% | 7.5 | 0.24 | [6.92 , 8.09] | ########## |
| 44 | 20 | 174 | Std B | Standard | 7.52 | | ########## | ########## | | ########## | 13.10% | | | | |

Telo_1

### 4.4 Aggregating Data

Once all samples have been assayed the total data can be aggregated to identify samples that need to be rerun and perform adjustments for plate to plate variation. When aggregating data each row corresponds to an individual replicate reaction and independent columns correspond to different features of that reaction as shown in **Figure 4.7**. The "Origin" column is a bookkeeping column that allows one to sort and always retain the original order of data as reflected within each assays and across assays when used in combination with the "Assay Number" column. The "Sample ID" column can be copied from the "Name" column in the RotorGene export shown in **Figure 4.5**. The "Estimated kb Telo" and "Estimated Genome Copies" columns correspond to the "Blanked Conc." columns in the RotorGene export template for the Telomere and Single Copy Gene runs as described in **Step 4.3** and shown in **Figure 4.6**. The "Filtered" counterparts to these columns contain replicate values with outliers removed as described in **Step 4.5**. The aTL (Raw) and aTL (Filtered) values are calculated as described in **Step 4.6** and the aTL (Adjusted) values are calculated as described in **Step 4.7**.

**Figure 4.7: Data Aggregation Template for aTL Calculations Using RotorGene Data**

| A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Origin | Sample ID | Assay Number | Estimated kb Telo | CV (kb Telo) | Estimated kb Telo (Filtered) | CV (Filtered kb Telo) | Estimated Genome Copies | CV (Estimated Genome Copies) | Estimated Genome Copies (Filtered) | CV (Filtered Genome Copies) | aTL (Raw) | aTL (Filtered) | aTL (Adjusted) |
| 1 | 1_PBMC | 1 | 1.31E+06 | 24.43 | 1.31E+06 | 7.08 | 2.44E+03 | 3.63 | 2.44E+03 | 53 | 6.66 | 5.74 | 5.53 |
| 2 | 1_PBMC | 1 | 1.18E+06 | | 1.18E+06 | | 2.38E+03 | | 2.38E+03 | | | | |
| 3 | 1_PBMC | 1 | 1.85E+06 | | | | 2.27E+03 | | 2.27E+03 | | | | |
| 4 | 7_PBMC | 1 | 2.59E+06 | 12.61 | 2.59E+06 | 12.61 | 2.65E+03 | 6.48 | 2.65E+03 | 6.48 | 9.74 | 9.74 | 9.40 |
| 5 | 7_PBMC | 1 | 2.01E+06 | | 2.01E+06 | | 2.38E+03 | | 2.38E+03 | | | | |
| 6 | 7_PBMC | 1 | 2.33E+06 | | 2.33E+06 | | 2.69E+03 | | 2.69E+03 | | | | |
| 7 | 8_PBMC | 1 | 2.16E+06 | 4.61 | 2.16E+06 | 4.61 | 2.00E+03 | 12.11 | 2.00E+03 | 12.11 | 12.57 | 12.57 | 12.12 |
| 8 | 8_PBMC | 1 | 2.11E+06 | | 2.11E+06 | | 1.82E+03 | | 1.82E+03 | | | | |
| 9 | 8_PBMC | 1 | 1.97E+06 | | 1.97E+06 | | 1.57E+03 | | 1.57E+03 | | | | |
| 10 | 9_PBMC | 1 | 2.09E+06 | 7.16 | 2.09E+06 | 7.16 | 1.59E+03 | 1.28 | 1.59E+03 | 1.28 | 13.93 | 13.93 | 13.44 |
| 11 | 9_PBMC | 1 | 1.84E+06 | | 1.84E+06 | | 1.55E+03 | | 1.55E+03 | | | | |
| 12 | 9_PBMC | 1 | 2.09E+06 | | 2.09E+06 | | 1.55E+03 | | 1.55E+03 | | | | |
| 13 | 13_PBMC | 1 | 1.90E+06 | 24.75 | | 6.29 | 2.03E+03 | 5.80 | 2.03E+03 | 5.80 | 7.76 | 6.66 | 6.43 |
| 14 | 13_PBMC | 1 | 1.22E+06 | | 1.22E+06 | | 1.99E+03 | | 1.99E+03 | | | | |
| 15 | 13_PBMC | 1 | 1.33E+06 | | 1.33E+06 | | 2.22E+03 | | 2.22E+03 | | | | |
| 16 | 14_PBMC | 1 | 1.90E+06 | 19.32 | 1.90E+06 | 5.17 | 2.63E+03 | 2.59 | 2.63E+03 | 2.59 | 9.40 | 8.37 | 8.07 |
| 17 | 14_PBMC | 1 | 2.70E+06 | | | | 2.57E+03 | | 2.57E+03 | | | | |
| 18 | 14_PBMC | 1 | 2.05E+06 | | 2.05E+06 | | 2.49E+03 | | 2.49E+03 | | | | |
| 19 | 16_PBMC | 1 | 1.43E+06 | 2.12 | 1.43E+06 | 2.12 | 2.03E+03 | 3.28 | 2.03E+03 | 3.28 | 7.62 | 7.62 | 7.35 |
| 20 | 16_PBMC | 1 | 1.38E+06 | | 1.38E+06 | | 1.92E+03 | | 1.92E+03 | | | | |
| 21 | 16_PBMC | 1 | 1.38E+06 | | 1.38E+06 | | 2.03E+03 | | 2.03E+03 | | | | |
| 22 | 26_PBMC | 1 | 3.22E+06 | 7.73 | 3.22E+06 | 7.73 | 2.85E+03 | 8.79 | 2.85E+03 | 8.79 | 13.61 | 13.61 | 13.12 |
| 23 | 26_PBMC | 1 | 3.53E+06 | | 3.53E+06 | | 2.40E+03 | | 2.40E+03 | | | | |
| 24 | 26_PBMC | 1 | 3.03E+06 | | 3.03E+06 | | 2.56E+03 | | 2.56E+03 | | | | |
| 25 | 28_PBMC | 1 | 1.67E+06 | 22.28 | | 0.92 | 1.73E+03 | 4.65 | 1.73E+03 | 4.65 | 8.64 | 7.53 | 7.26 |
| 26 | 28_PBMC | 1 | 1.16E+06 | | 1.16E+06 | | 1.69E+03 | | 1.69E+03 | | | | |
| 27 | 28_PBMC | 1 | 1.15E+06 | | 1.15E+06 | | 1.58E+03 | | 1.58E+03 | | | | |

### 4.5 Reviewing Data for Outliers and Determining Reruns

To avoid overmanipulating the data, only those samples with a replicate CV greater than 15% are inspected for outliers. In these instances, outliers are determined following a similar rule as described for dropping outliers for replicate Ct values of standards. Specifically, **a replicate is dropped as an outlier when both the following conditions are met 1) the CV across replicate reactions is greater than 15% and 2) the absolute distance between the estimated kb telo or genome copies for the outlier replicate and the closest replicate kb telo or genome copies is greater than twice the distance between the remaining two replicates. In instances where the CV across replicate estimates is greater than 15% without a clear outlier all replicates are retained and that sample is re-assayed a second time.**

### 4.6 Calculating aTL Estimates

Final aTL estimates calculated in Columns L and M correspond to estimates generated using the raw data and filtered data. These estimates are calculated using the formula below, where the Average kb Telo and Average Genome Copies represent the average across triplicate values within a given run (or duplicate values if an outlier replicate was dropped).

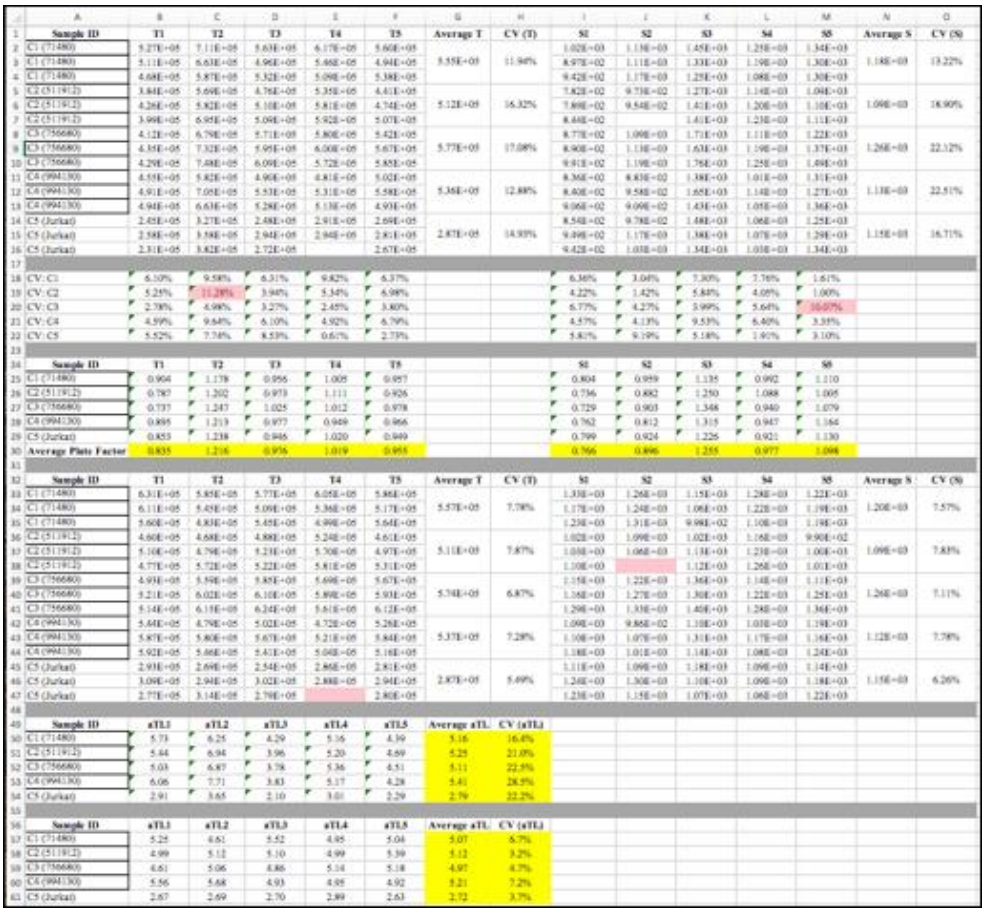
$$\boldsymbol{aTL} = \frac{\left(\frac{Average\ kb\ Telo}{Average\ Genome\ Copies}\right)}{92}$$

### 4.7 Adjusting for Plate-to-Plate Variation

Five control samples run on every plate are used to control for plate-to-plate variation. For each assay, the estimated kb telomeric DNA and estimated genome copy number are divided by the average kb telomeric DNA and average genome copy number for the same control sample across all assays to get a normalizing factor for that sample on a given plate. This is repeated across all five control samples to generate an average normalizing factor for a given assay (row 32), and repeated again across all assays to generate normalizing factors for each assay. An example layout to generate normalizing factors across 5 assays is illustrated in in **Figure 4.8**. Note how the average aTL values for each control sample exhibit minimal change before (column G, rows 50-54) and after normalizing (column G, rows 57-61), yet the CV deceased from an average of 22% to an average of 5%. The estimated kb telomeric DNA and estimated genome copy number values for all samples on a given plate are divided by the normalizing factor for that plate to generate the final adjusted aTL values used in analyses.

We have conducted various tests for how to best optimize this normalization process, for example testing whether to normalize at the level of estimated telomeric content/genome copy number increases reproducibility. Overall, we observed minimal impact of the plate-level versus assay-level normalization approach and opted to normalize at the level of estimated kb telomeric DNA and estimated genome copies as they do not always vary in the same direction. We have also tested the impact of plate-to-plate adjustments on the assay ICC. In a test of 20 samples assayed 4 times, the ICC of raw values not adjusted for plate-to-plate variation was 0.404, which increased to 0.655 when a fixed factor for assay was added to the model. By comparison the ICC for adjusted aTL values was 0.833 and remained relatively unchanged (0.830) when a fixed factor for assay was added to the model.

## Figure 4.8 Example Layout for Plate-to-Plate Adjustments



| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | **Sample ID** | **T1** | **T2** | **T3** | **T4** | **T5** | **Average T** | **CV (T)** | **S1** | **S2** | **S3** | **S4** | **S5** | **Average S** | **CV (S)** |
| 2 | C1 (71480) | 5.27E+05 | 7.11E+05 | 5.63E+05 | 6.17E+05 | 5.60E+05 | | | 1.02E+03 | 1.13E+03 | 1.45E+03 | 1.25E+03 | 1.34E+03 | | |
| 3 | C1 (71480) | 5.11E+05 | 6.63E+05 | 4.96E+05 | 5.46E+05 | 4.94E+05 | 5.55E+05 | 11.94% | 8.97E+02 | 1.11E+03 | 1.33E+03 | 1.19E+03 | 1.30E+03 | 1.18E+03 | 13.22% |
| 4 | C1 (71480) | 4.68E+05 | 5.87E+05 | 5.32E+05 | 5.09E+05 | 5.38E+05 | | | 9.42E+02 | 1.17E+03 | 1.25E+03 | 1.08E+03 | 1.30E+03 | | |
| 5 | C2 (511912) | 3.84E+05 | 5.69E+05 | 4.76E+05 | 5.35E+05 | 4.41E+05 | | | 7.82E+02 | 9.73E+02 | 1.27E+03 | 1.14E+03 | 1.04E+03 | | |
| 6 | C2 (511912) | 4.26E+05 | 5.82E+05 | 5.10E+05 | 5.81E+05 | 4.74E+05 | 5.12E+05 | 16.32% | 7.89E+02 | 9.54E+02 | 1.41E+03 | 1.20E+03 | 1.10E+03 | 1.09E+03 | 18.90% |
| 7 | C2 (511912) | 3.99E+05 | 6.95E+05 | 5.09E+05 | 5.92E+05 | 5.07E+05 | | | 8.44E+02 | | 1.41E+03 | 1.21E+03 | 1.11E+03 | | |
| 8 | C3 (756680) | 4.12E+05 | 6.79E+05 | 5.71E+05 | 5.80E+05 | 5.42E+05 | | | 8.77E+02 | 1.09E+03 | 1.71E+03 | 1.11E+03 | 1.22E+03 | | |
| 9 | C3 (756680) | 4.35E+05 | 7.32E+05 | 5.95E+05 | 6.00E+05 | 5.67E+05 | 5.77E+05 | 17.08% | 8.90E+02 | 1.13E+03 | 1.63E+03 | 1.19E+03 | 1.37E+03 | 1.26E+03 | 22.12% |
| 10 | C3 (756680) | 4.29E+05 | 7.48E+05 | 6.09E+05 | 5.72E+05 | 5.85E+05 | | | 9.91E+02 | 1.19E+03 | 1.76E+03 | 1.25E+03 | 1.49E+03 | | |
| 11 | C4 (994130) | 4.55E+05 | 5.82E+05 | 4.90E+05 | 4.81E+05 | 5.02E+05 | | | 8.36E+02 | 8.83E+02 | 1.38E+03 | 1.01E+03 | 1.31E+03 | | |
| 12 | C4 (994130) | 4.91E+05 | 7.05E+05 | 5.53E+05 | 5.31E+05 | 5.58E+05 | 5.36E+05 | 12.88% | 8.40E+02 | 9.58E+02 | 1.65E+03 | 1.14E+03 | 1.27E+03 | 1.13E+03 | 22.51% |
| 13 | C4 (994130) | 4.94E+05 | 6.63E+05 | 5.28E+05 | 5.13E+05 | 4.93E+05 | | | 9.06E+02 | 9.09E+02 | 1.43E+03 | 1.05E+03 | 1.36E+03 | | |
| 14 | C5 (Jurkat) | 2.45E+05 | 3.27E+05 | 2.48E+05 | 2.91E+05 | 2.69E+05 | | | 8.54E+02 | 9.78E+02 | 1.48E+03 | 1.06E+03 | 1.25E+03 | | |
| 15 | C5 (Jurkat) | 2.58E+05 | 3.58E+05 | 2.94E+05 | 2.84E+05 | 2.81E+05 | 2.87E+05 | 14.93% | 9.49E+02 | 1.17E+03 | 1.38E+03 | 1.07E+03 | 1.29E+03 | 1.15E+03 | 16.71% |
| 16 | C5 (Jurkat) | 2.31E+05 | 3.62E+05 | 2.72E+05 | | 2.67E+05 | | | 9.42E+02 | 1.03E+03 | 1.34E+03 | 1.03E+03 | 1.34E+03 | | |
| 17 | | | | | | | | | | | | | | | |
| 18 | CV: C1 | 6.10% | 9.58% | 6.31% | 9.82% | 6.37% | | | 6.36% | 3.04% | 7.30% | 7.76% | 1.61% | | |
| 19 | CV: C2 | 5.25% | 11.28% | 3.94% | 5.34% | 6.98% | | | 4.22% | 1.42% | 5.84% | 4.05% | 1.00% | | |
| 20 | CV: C3 | 2.78% | 4.98% | 3.27% | 2.45% | 3.80% | | | 6.77% | 4.27% | 3.99% | 5.64% | 10.07% | | |
| 21 | CV: C4 | 4.59% | 9.64% | 6.10% | 4.92% | 6.79% | | | 4.57% | 4.13% | 9.53% | 6.40% | 3.35% | | |
| 22 | CV: C5 | 5.52% | 7.74% | 8.53% | 0.61% | 2.73% | | | 5.81% | 9.19% | 5.18% | 1.91% | 3.10% | | |
| 23 | | | | | | | | | | | | | | | |
| 24 | **Sample ID** | **T1** | **T2** | **T3** | **T4** | **T5** | | | **S1** | **S2** | **S3** | **S4** | **S5** | | |
| 25 | C1 (71480) | 0.904 | 1.178 | 0.956 | 1.005 | 0.957 | | | 0.804 | 0.959 | 1.135 | 0.992 | 1.110 | | |
| 26 | C2 (511912) | 0.787 | 1.202 | 0.973 | 1.111 | 0.926 | | | 0.736 | 0.882 | 1.250 | 1.088 | 1.005 | | |
| 27 | C3 (756680) | 0.737 | 1.247 | 1.025 | 1.012 | 0.978 | | | 0.729 | 0.903 | 1.348 | 0.940 | 1.079 | | |
| 28 | C4 (994130) | 0.895 | 1.213 | 0.977 | 0.949 | 0.966 | | | 0.762 | 0.812 | 1.315 | 0.947 | 1.164 | | |
| 29 | C5 (Jurkat) | 0.853 | 1.238 | 0.946 | 1.020 | 0.949 | | | 0.799 | 0.924 | 1.225 | 0.921 | 1.130 | | |
| 30 | **Average Plate Factor** | 0.835 | 1.216 | 0.976 | 1.019 | 0.955 | | | 0.766 | 0.896 | 1.255 | 0.977 | 1.098 | | |
| 31 | | | | | | | | | | | | | | | |
| 32 | **Sample ID** | **T1** | **T2** | **T3** | **T4** | **T5** | **Average T** | **CV (T)** | **S1** | **S2** | **S3** | **S4** | **S5** | **Average S** | **CV (S)** |
| 33 | C1 (71480) | 6.31E+05 | 5.85E+05 | 5.77E+05 | 6.05E+05 | 5.86E+05 | | | 1.33E+03 | 1.26E+03 | 1.15E+03 | 1.24E+03 | 1.22E+03 | | |
| 34 | C1 (71480) | 6.11E+05 | 5.45E+05 | 5.09E+05 | 5.36E+05 | 5.17E+05 | 5.57E+05 | 7.78% | 1.17E+03 | 1.24E+03 | 1.06E+03 | 1.22E+03 | 1.19E+03 | 1.20E+03 | 7.57% |
| 35 | C1 (71480) | 5.60E+05 | 4.83E+05 | 5.45E+05 | 4.99E+05 | 5.64E+05 | | | 1.23E+03 | 1.31E+03 | 9.98E+02 | 1.10E+03 | 1.19E+03 | | |
| 36 | C2 (511912) | 4.60E+05 | 4.68E+05 | 4.88E+05 | 5.24E+05 | 4.61E+05 | | | 1.02E+03 | 1.09E+03 | 1.02E+03 | 1.16E+03 | 9.90E+02 | | |
| 37 | C2 (511912) | 5.10E+05 | 4.79E+05 | 5.21E+05 | 5.30E+05 | 4.97E+05 | 5.11E+05 | 7.87% | 1.03E+03 | 1.06E+03 | 1.13E+03 | 1.23E+03 | 1.00E+03 | 1.09E+03 | 7.85% |
| 38 | C2 (511912) | 4.77E+05 | 5.72E+05 | 5.22E+05 | 5.81E+05 | 5.31E+05 | | | 1.10E+03 | | 1.12E+03 | 1.26E+03 | 1.01E+03 | | |
| 39 | C3 (756680) | 4.95E+05 | 5.59E+05 | 5.85E+05 | 5.69E+05 | 5.67E+05 | | | 1.15E+03 | 1.22E+03 | 1.36E+03 | 1.14E+03 | 1.11E+03 | | |
| 40 | C3 (756680) | 5.21E+05 | 6.02E+05 | 6.10E+05 | 5.89E+05 | 5.93E+05 | 5.74E+05 | 6.87% | 1.16E+03 | 1.27E+03 | 1.30E+03 | 1.22E+03 | 1.25E+03 | 1.26E+03 | 7.11% |
| 41 | C3 (756680) | 5.14E+05 | 6.15E+05 | 6.24E+05 | 5.61E+05 | 6.12E+05 | | | 1.29E+03 | 1.33E+03 | 1.40E+03 | 1.24E+03 | 1.36E+03 | | |
| 42 | C4 (994130) | 5.44E+05 | 4.79E+05 | 5.02E+05 | 4.72E+05 | 5.26E+05 | | | 1.09E+03 | 9.86E+02 | 1.38E+03 | 1.03E+03 | 1.19E+03 | | |
| 43 | C4 (994130) | 5.87E+05 | 5.80E+05 | 5.67E+05 | 5.21E+05 | 5.84E+05 | 5.37E+05 | 7.28% | 1.30E+03 | 1.07E+03 | 1.31E+03 | 1.17E+03 | 1.16E+03 | 1.12E+03 | 7.78% |
| 44 | C4 (994130) | 5.92E+05 | 5.66E+05 | 5.41E+05 | 5.04E+05 | 5.16E+05 | | | 1.18E+03 | 1.01E+03 | 1.14E+03 | 1.08E+03 | 1.24E+03 | | |
| 45 | C5 (Jurkat) | 2.93E+05 | 2.69E+05 | 2.54E+05 | 2.86E+05 | 2.81E+05 | | | 1.11E+03 | 1.09E+03 | 1.18E+03 | 1.09E+03 | 1.14E+03 | | |
| 46 | C5 (Jurkat) | 3.09E+05 | 2.94E+05 | 3.02E+05 | 2.88E+05 | 2.94E+05 | 2.87E+05 | 5.69% | 1.24E+03 | 1.30E+03 | 1.10E+03 | 1.09E+03 | 1.18E+03 | 1.15E+03 | 6.26% |
| 47 | C5 (Jurkat) | 2.77E+05 | 3.14E+05 | 2.79E+05 | | 2.80E+05 | | | 1.23E+03 | 1.15E+03 | 1.07E+03 | 1.06E+03 | 1.22E+03 | | |
| 48 | | | | | | | | | | | | | | | |
| 49 | **Sample ID** | **aTL1** | **aTL2** | **aTL3** | **aTL4** | **aTL5** | **Average aTL** | **CV (aTL)** | | | | | | | |
| 50 | C1 (71480) | 5.71 | 6.25 | 4.29 | 5.36 | 4.39 | 5.16 | 16.4% | | | | | | | |
| 51 | C2 (511912) | 5.44 | 6.94 | 3.96 | 5.20 | 4.69 | 5.25 | 21.0% | | | | | | | |
| 52 | C3 (756680) | 5.03 | 6.87 | 3.78 | 5.36 | 4.51 | 5.11 | 22.5% | | | | | | | |
| 53 | C4 (994130) | 6.06 | 7.71 | 3.83 | 5.17 | 4.28 | 5.41 | 28.9% | | | | | | | |
| 54 | C5 (Jurkat) | 2.91 | 3.65 | 2.10 | 3.01 | 2.29 | 2.79 | 22.2% | | | | | | | |
| 55 | | | | | | | | | | | | | | | |
| 56 | **Sample ID** | **aTL1** | **aTL2** | **aTL3** | **aTL4** | **aTL5** | **Average aTL** | **CV (aTL)** | | | | | | | |
| 57 | C1 (71480) | 5.25 | 4.61 | 5.52 | 4.95 | 5.04 | 5.07 | 6.7% | | | | | | | |
| 58 | C2 (511912) | 4.99 | 5.12 | 5.10 | 4.99 | 5.39 | 5.12 | 3.2% | | | | | | | |
| 59 | C3 (756680) | 4.61 | 5.06 | 4.86 | 5.14 | 5.18 | 4.97 | 4.7% | | | | | | | |
| 60 | C4 (994130) | 5.56 | 5.68 | 4.93 | 4.95 | 4.92 | 5.21 | 7.2% | | | | | | | |
| 61 | C5 (Jurkat) | 2.67 | 2.69 | 2.70 | 2.89 | 2.63 | 2.72 | 3.7% | | | | | | | |

## 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 | Removed the use of plasmid pBR322 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. |
| November 12, 2021 | WJH | Changed procedure for aliquoting and storage of primers such that primer pairs (i.e., TPR+TPF & IFNB1R+IFNB1F) are stored together in single aliquots instead of in separate aliquots. This removes half of the potential variability across plates due to small differences between primer aliquots. The volumes and structure of the mastermix composition in Table 3.2.3 were adjusted accordingly. |
| November 12, 2021 | WJH | Modified the overall plate layout in Figure 1 to remove the off-target reactions for control samples. Previously, each plate consisted of 91 reactions for the target gene and 9 reactions of the off-target gene (e.g., TELO plates had 90x TELO reactions and 9x IFNB1 reactions for 3 control samples). This previous format was done with the intention of controlling for plate-to-plate variation in between TELO and IFNB1 plates of the same assay. However, this was not practical for the aTL assay because we could not calculate estimated genome copy number on the TELO plate because that plate did not contain the IFNB1 standards. Similarly, we could not calculate kb telomeric DNA on the IFNB1 plate because that plate lacked the TELO standards. Furthermore, applying any control factors generated by the off-target reactions to the main reactions would be inappropriate since the largest contributor to plate-to-plate variation, differences in mastermix composition, were not conserved between these two sets. As a result, the overall layout for each plate now consists of 6 standards, 22 analytical samples, 5 controls, and 1 no-template blank. |
| December 10, 2021 | WJH | Added Section 4 to describe analysis pipeline for calculating aTL values using data from the RotorGene instrument. |
| December 13, 2021 | WJH | Changed composition of standard reaction wells such that each standard reaction was composed of 6uL standard and 14uL mastermix instead of 4uL standard, 2uL water, and 14uL mastermix. Changed standard construction (Steps 3.1 – 3.1.5) to reflect changes in standard concentration. Note: standard concentrations were modified such that expected kb telomeric DNA and genome copy numbers remained the same as previous implementations. |
| January 11, 2022 | WJH | Modified caption in table of primers in Section 2 to reflect change in procedure to order primers as premixed primer pairs (RxnReady® Primer Pool) instead of ordering primers as individual oligomers. Modified step 3.2.2 of PCR setup to reflect premixed primer pairs, which require direct aliquoting instead of diluting and combining as was done with individual primer oligos. |
| July 20, 2022 | WJH | Modified Figure 1 to reflect updated plate layout wherein 5 control samples are run in wells 1-15 instead of in wells 85-99. This change was implemented because we observed increased variability across replicate Ct values in the first 15 wells and wanted to minimize the impact such variability had on estimates for standards. |

## 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.
3. Ramakers, C., Ruijter, J. M., Lekanne Deprez, R. H., & Moorman, A. F. M. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters*, *339*(1), 62-66.