TELOMERE LENGTH MEASUREMENT USING QPCR

1. PURPOSE & GENERAL OVERVIEW

This protocol describes measurement of absolute telomere length (aTL) using qPCR. This protocol was adapted by the Shalev Lab from previously published methods by Cawthon and O'Callaghan[1,2]. This method 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 telomeric sequence per cell. A second division by 92 (23 chromosomal pairs with a telomere on each end) reports telomere length per telomere (in bp values). Plasmid DNA (pBR322) is added to the oligomer standard to maintain a constant amount of DNA similar to that found in each sample.

2. MATERIALS/REAGENTS/EQUIPMENT

2.1 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'-GGA CTG GAC AAT TGC TTC AAG -3'
IFNB1-R	5'- CCT TTC ATA TGC AGT ACA TTA G -3'

Primers are purchased from IDT (www.idtdna.com) in lab-ready from (HPLC purified, 100uM in IDTE Buffer pH 8.0).

2.2 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 TTA GGG TTA GGG TTA GGG-3'
IFNB1 Standard	
Sense	5'- CCT TTC ATA TGC AGT ACA TTA GCC ATC AGT CAC TTA AAC AGC ATC TGC TGG TTG AAG AAT GCT TGA AGC AAT TGT CCA GTC C -3'
Antisense	5'-GGA CTG GAC AAT TGC TTC AAG CAT TCT TCA ACC AGC AGA TGC TGT TTA AGT GAC TGA TGG CTA ATG TAC TGC ATA TGA AAG G-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.

2.3 Reagents and supplies

Material	**Vendor**	**Cat#**
SYBR Green PCR Kit	Qiagen	204145
Uracil-DNA glycosylase (UNG)	Thermo Scientific	FEREN0362
pBR322 DNA Plasmid (0.5 ug/uL)	Thermo Scientific	FERSD0041
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
15mL conical tube	VWR	89126-798
50mL conical tube	VWR	89126-802
2mL cryovial	VWR	10018-754
1.5mL Eppendorf tube	VWR	20170-038
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

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 made by making a 0.15ng/ul dilution of the telomere oligomer standard to measure concentration. A 10-fold dilution is performed to generate a 0.015ng/ul Standard A. This Standard A corresponds to containing 60pg DNA when 4uL is used in the qPCR assay. The number of telomere repeats in Standard A is calculated by:

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

Molecules in Standard = DNA per reaction / Weight of one molecule = $\frac{60.00\ x\ 10^{-12} g}{8.60\ x\ 10^{-20} g} = 6.98\ x\ 10^{8}.$

Each oligomer is 84bp in length. Thus, telomere Standard A corresponds to $6.98\ x\ 10^{8} * 84 = 5.86\ x\ 10^{10} bp = 5.86\ x\ 10^{10} bp$ or 5.86 x 10^7 kb telomeric DNA. Ten-fold serial dilutions are performed to generate standards from 5.86 x 10^7 kb to 5.86 x 10^2 kb.

Single Copy Gene Standard

The first single copy gene standard is made by making a 0.05ng/ul dilution of the duplex oligomers. A 100-fold dilution is performed to generate a 0.0005ng/uL Single Copy Standard 1. 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:

Weight of one molecule = Molecular Weight/Avogadro's number = $\frac{50537.8 g}{6.022\ x\ 10^{23}} = 8.39\ x\ 10^{-20} g.$

Molecules in Standard 1= DNA per reaction / Weight of one molecule = $\frac{2.00\ x\ 10^{-12}}{8.39\ x\ 10^{-20}} = 2.38\ x\ 10^{7}.$

Because there are two copies of this gene, the:

Diploid Copy Number = (Concentration / Weight of one molecule)/ 2 = $2.38\ x\ 10^{7}$/2 = 1.19 x 10^7.

Ten-fold serial dilutions are performed with this Standard 1 to generate concentrations from 1.19 x 10^7 to 1.19 x 10^1 for the standard curve.

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 100uL 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 10uL *factory stock* with 990uL 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 of *working stock* to use and add 0.25x TE to for a final 2mL volume for telomere Standard A. *an additional 1/10 dilution of the *working stock* may be necessary*

$$Volume\ TELO\ Working\ Stock\ (uL) = \frac{(1.50\ x10^{-2}\ ng/uL\ )(2000\ uL)}{Concentration\ Working\ Stock\ (\frac{ng}{uL})}$$

3.1.4b Use equation below to calculate volume of *working stock* to use and to add 0.25x TE for a final 2mL volume for the Single Copy Standard 1. *an additional 1/10 dilution of the *working stock* may be necessary*

$$Volume\ IFNB1\ Working\ Stock\ (uL) = \frac{(5.00\ x10^{-4}\ ng/uL\ )(2000\ uL)}{Concentration\ Working\ Stock\ (\frac{ng}{uL})}$$

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 detailed below are generally suitable for most applications.

Standard Range for Telomere Standards

Standard	ng/uL	pg DNA/reaction	Kb telo sequence
A	1.50e-02	60	5.86e+07
B	1.50e-03	6.0	5.86e+06
C	1.50e-04	0.6	5.86e+05
D	1.50e-05	0.06	5.86e+04
E	1.50e-06	0.006	5.86e+03
F	1.50e-07	0.0006	5.86e+02
G	1.50e-08	0.00006	5.86e+01

Standard Range for IFNB1 Standards

Standard	ng/uL	pg DNA/reaction	Genome Copies
1	5.0e-04	2	1.19e+07
2	5.0e-05	0.2	1.19e+06
3	5.0e-06	0.02	1.19e+02
4	5.0e-07	0.002	1.19e+04
5	5.0e-08	0.0002	1.19e+03
6	5.0e-09	0.00002	1.19e+02
7	5.0e-10	0.000002	1.19e+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 0.5 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 1100uL total volume by adding 110uL factory stock to 990uL 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 Plasmid Working Stock

Construct working stock of pBR322 plasmid. Factory item is delivered at a concentration of 500 ng/uL. Create an initial dilution at 10 ng/uL by combining 20uL factory stock with 980uL 0.25x TE buffer. Perform an additional 1/10 dilution to finalize plasmid working stock at 1 ng/uL. Plasmid working stock can be stored in aliquots at -20°C for up to one year. When running multiple assays several days in a row, plasmid working stock can be stored at 4°C to avoid freeze thaws. Plasmid working stock stored at 4°C should be used within a month.

3.2.4 PCR Mastermix

Construct PCR mastermix appropriate for the number of samples on a given plate. Various compositions are detailed below.

PCR Mastermix Construction

Reagent	1x (uL)	5x (uL)	50x (uL)	105x (uL)
SYBR Green PCR Mix	10	50	500	1050
10uM TPF/IFNB1-F	0.2	1.0	10	21
10uM TPR/IFNB1-R	0.2	1.0	10	21
UNG (1U/uL)	0.2	1.0	10	21
UltraPure PCR Water	3.4	17	170	357
Final Volume	14	70	700	1470

3.3 Plate Loading and PCR Cycling

3.3.1 Open the in-house Qiagility program template titled "Telomere_6standards_3controls" and load Rotor-disc 100 rotor with 100 well plates within the robotic pipette according to the layout detailed on the program. Once all standards, samples, controls, plasmid, 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. Plasmid DNA is added to standard wells to keep total ng DNA near 3 ng 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 (0.5 ng/uL)	-	6 uL	-
Standard A-G/1-10	4 uL	-	-
Plasmid (1 ng/uL)	2 uL	-	-
UltraPure H_20	-	-	6 uL
Total Volume	20 uL	20 uL	20 uL

3.3.2 Once the robot has completed loading the rotor disk (~40 min), remove the entire 100-well ring 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 block and seal into 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 Qiagility block and load onto the RotorGene Q Rotor disc. Add locking ring to and load onto the RotorGene Q for PCR cycling at the conditions below.

PCR Cycling

Hold at 50°C for 2 mins
Hold at 95°C for 15 seconds
Denature at 95°C for 15 sec; Anneal/extend at 60°C for 1 min; 45 cycles
Melt curve generated ramping from 50°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 and an IFNB1 qPCR assay. 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).