[January 7, 2020]

TELOMERE LENGTH MEASUREMENT USING QPCR

1. PURPOSE:

This protocol describes telomere length measurement using qPCR, adapted by Jue Lin from Dr. Richard M. Cawthon's method [1, 2].

2. MATERIALS/REAGENTS/EQUIPMENT

2.1 Primer sequences

Primer	Sequence
Tel 1b	5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'
Tel 2b	5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'
Hbg 1	5'-GCTTCTGACACAACTGTGTTCACTAGC-3'
Hbg 2	5'-CACCAACTTCATCCACGTTCACC-3'

All primers were purchased from IDT (www.idtdna.com) in standard desalted form.

2.2 Reagents and supplies

Material	Vendor	Cat#
Platinum Taq polymerase	Invitrogen	10966083, 5000 Units
E. Coli DNA	Sigma-Aldrich	D4889-5UN
PCR-grade dNTPs	Thermo-Fisher	R0186
Human genomic DNA from buffy coat	Sigma-Aldrich	11691112001
SYBR® Green I Nucleic Acid Gel Stain	Invitrogen	S7585
DMSO	Sigma-Aldrich	154938
15 ml conical tube	varies	varies
50 ml conical tube	varies	varies
5 ml conical tube	varies	varies
1.7 ml eppendorf tube (presterilized)	varies	varies
2 ml eppendorf tube (presterilized)	varies	varies
Autoclaved Millipore water	Lin lab	N/A
10 ml sterile serological pipette	Fisher	13-676-10J
25 ml sterile serological pipette	Fisher	13-676-10K
500 ml and 1000 ml autoclaved bottles	Lin lab	N/A
96-Well Skirted PCR Plates	BioRad	HSP-9901
Microseal® 'F' Foil	BioRad	MSF-1001
Plate roller	varies	varies
384 well assay plate with seals	Bio-Rad	HSR4805
96 well metal block	Diversified Biotech	CHAM-1000
384 well metal block	Diversified Biotech	CHAM-3840
0.5 ml thin wall PCR tube	Molecular Bioproducts	3430

P1000 pipetteman and filter tips	varies	varies
P200 pipetteman and filter tips	varies	varies
P20 pipetteman and filter tips	varies	varies
P20 tips for Biomek 3000	VWR	89204-790

2.3. Equipment

-Sorvall Legend XTR tabletop centrifuge
- Agilgent SureCycler 8800 PCR machine
-BioRad C1000 thermocycler
-Biomek 3000 liquid handler
-Roche LC480 realtime PCR machine
-Eppendorf microfuge, Model 5415D
-Tomy Capsulefuge, Model PMC-860
- BD Clay Adams™ Nutator Mixer
–80ºC freezer
-NanoDrop 2000C

3. EXPERIMENTAL PROCEDURES

3.1. Preparation of Reagents

3.1.1. **E. Coli DNA stock**

3.1.1.1. Resuspend the entire bottle of E. coli DNA in 16.67 ml PCR grade H_2O with the intended concentration of 600ng/µl.

3.1.1.2. Transfer to a 50 ml conical tube, rotate on a Nutator at room temperature overnight.

3.1.1.3. Measure OD260/OD280 on NanoDrop to determine the DNA concentration. Unused E. Coli DNA stock can be stored at -20 ºC for at least 1 year.

3.1.2. DI buffer

3.1.2.1. Calculate the volume of E. Coli DNA stock need to make 1 L DI buffer

3.1.2.2. Use a 1000 ml glass bottle, add the following:

100 ml 10x PCR buffer from the Platinum Taq polymerase kit
E. Coli DNA stock
PCR grade water to bring the final volume to be 1 L
Mix thoroughly by swirling the bottle for 30-40 times.

3.1.2.4. Store DI buffer in aliquots of 50 ml in 50 ml conical tubes at -80 ºC. Good for at least a year at -80 ºC.

3.1.2.5. Further aliquot DI buffer into 10 ml in 15 ml conical tubes. Store the 10 ml aliquots at -20 ºC. Use within 3 months.

3.1.3. U mix

3.1.3.1. Use a 1000 ml glass bottle, add each component according to Table 1. Mix thoroughly by swirling the bottle for 30-40 times.

Table 1. U Mix components.

	amount	unit
Autoclaved Millipore water	698.8	ml
10XPCR buffer	150	ml

MgCl2 (50 mM)	66	ml
dNTPS (25 mM each of A, C, G and T)	17.6	ml
DMSO	22	ml
TOTAL volume	954.4	ml

3.1.3.2. Store U Mix in aliquots of 40 ml in 50 ml conical tubes at -80 ºC. Good for at least a year at -80 ºC.
3.1.3.3. Further aliquot U Mix into 1.5 ml in 2 ml eppendorf tubes. Store the 1.5 ml aliquots at -80 ºC. Use within 3 months.

3.1.4. T mix

3.1.4.1. Resuspend Tel1b and Tel2b primers in autoclaved Millipore water to 1mM.
3.1.4.2. Dilute Tel 1b and Tel 2b primers to 100 µM with autoclaved Millipore water.
3.1.4.3. Use a 500 ml glass bottle, add each component according to Table 2. Mix thoroughly by swirling the bottle for 30-40 times.

Table 2. T Mix components

	amount	unit
Autoclaved Millipore water	418	ml
Tel 1b Primer (100 µM)	2.2	ml
Tel 2b Primer (100 µM)	19.8	ml
TOTAL volume	440	ml

3.1.3.4. Store T Mix in aliquots of 40 ml in 50 ml conical tubes at -80 ºC. Good for at least a year at -80 ºC.
3.1.4.5. Further aliquot T Mix into 1ml in 1.5 ml eppendorf tubes. Store the 1 ml aliquots at -80 ºC. Use within 3 months.

3.1.5. S mix

3.1.5.1. Resuspend Hbg 1 and Hbg 2 primers in autoclaved Millipore water to 1mM.
3.1.5.2. Dilute Hbg 1 and Hbg 2 primers to 100 µM with autoclaved Millipore water.
3.1.5.3. Use a 500 ml glass bottle, add each component according to Table 3. Mix thoroughly by swirling the bottle for 30-40 times.

Table 3. S Mix components

	amount	unit
Autoclaved Millipore water	374	ml
50 mM MgCl2	44	ml
Hbg 1 Primer (100 µM)	6.6	ml
Hbg 2 Primer (100 µM)	15.4	ml
TOTAL volume	440	ml

3.1.5.5. Store S Mix in aliquots of 40 ml in 50 ml conical tubes at -80 ºC. Good for at least a year at -80 ºC.
3.1.5.6. Further aliquot S Mix into 1ml in 1.5 ml eppendorf tubes. Store the 1 ml aliquots at -80 ºC. Use within 3 months.

3.1.6. 10X SYBR green

NOTE: keep in aluminum foil as SYBR is light sensitive, minimize the time exposed to light. Make fresh daily

3.1.6.1. To make a 10X solution, add 5 µl of 10,000X SYBR Green I to 5 ml of autoclaved Millipore water in a 15 ml conical tube wrapped in aluminum foil. Keep on ice. Use it within the day.

3.2. Setting up DNA denaturation

3.2.1. Before start, from stock solutions, take out

One tube of DI buffer
Three tubes of U Mix
One tube of T Mix
One tube of S Mix

Thaw at room temperature for about 10 minutes for U, T and S mixes and about 20 minutes for DI buffer. Keep on ice once thawed.

3.2.2. Take out Reference DNA (Human Genomic DNA, stored at 4°C), 8 control DNAs (at 100 ng/µl) and sample DNA plate (stored at -80°C).
Thaw on ice. Gently tap the tubes to mix, briefly centrifuge (2000 rpm for 2 minutes) the tubes and put back on ice.

3.2.3. Record the reagent lot (SYBR Green, Taq polymerase, DI buffer, U, T and S mixes), reference DNA, control DNA lots in the Excel sheet.

3.2.4. *Prepare Biomek 3000 by* turning the three Peltier blocks on (switch is on the back). Put 96 well metal blocks on the Peltier blocks.

3.2.5. Open Biomek Software and open the program called "START HERE". Go to the pulldown menu, under "Instrument", click "Home Axis".

3.2.6. ***Prepare the sample DNA denaturation plate:***

3.2.6.1 Add 40 ul of DI buffer and 3 ul of DNA at 20-40 ng/ul by hand pipetting using a multichannel P50 pipettman.

3.2.6.2 After pipetting, seal the 96 well plate (DI+DNA samples) with PCR Foil seal (BioRad cat# MSF1001).

3.2.6.3. Vortex the plate for 10 seconds, spin at 2000 rpm for 2 minutes at 4°C in the Sorvall Tabletop centrifuge. Denature the DNA in a BioRad C1000 Thermocylcer at 96°C for 10 minutes, keep the DNA at 4°C.

3.2.6.4. When the Denature program is finished (lid temperature <85°C), put the plate on a 96 well metal block on ice right away. Wait 2 minutes, vortex the plate for 10 seconds, spin at 2000 rpm for 2 minutes at 4°C in the Sorvall Tabletop centrifuge. Put the denatured plate on the Peltier block.

3.2.7. ***Prepare control DNA and reference DNA***

3.2.7.1 Put three of 0.2 ml 8-strip PCR tubes on a 96 well metal block, label S1-S6 for standard (one strip) from left to right. Label C1-C8 x 2 strips for control DNA samples from left to right.

3.2.7.2 Reference DNA

Pipette 80 µl of DI buffer into tubes S1-S6, add 3µl of Reference DNA into S1 tube.

Pipette 50 µl of DI buffer control DNA tubes and add 1.2 µl of control DNAs into each tube. Close the tubes with 8-strip caps. Mix by vortexing 10 seconds, spin down the strips briefly in a Capsulefuge.

3.2.7.3. Denature the DNA in a Agilent SureCycler at 96°C for 10 minutes.

3.2.7.4. Take out the strips with denatured DNA and cool on a metal block on ice. Make sure that the caps are tight. Wait 3 minute and mix by vortexing 10 seconds. Spin down the strips briefly in a Tomy Capsulefuge. Leave the samples on metal block on ice.

3.3. ***Prepare the*** **standard curve: 6-point 3-fold serial dilution of the reference DNA**

3.3.1. tube, Pipette denatured DI buffer from tubes S2-S6 (from step 3.2.7.4) to a 1.5ml microcentrifuge, mix and keep on ice.

3.3.2. Take a new 8-strip tube, label as DS1-DS6. Pipette 50 µl of denatured DI from "DI" tube into DS2-DS7.

3.3.3. Transfer the contents of S1 to DS1.

3.3.4. Use a new tip, pipette 25 µl of the content of DS1 into DS2. Pipette up and down 8-10 times.

3.3.5. Use a new tip, pipette 25 µl of the content of DS2 into DS3. Pipette up and down 8-10 times.

3.3.6. Use a new tip, pipette 25 µl of the content of DS3 into DS4. Pipette up and down 8-10 times.

3.3.7. Use a new tip, pipette 25 µl of the content of DS4 into DS5. Pipette up and down 8-10 times.

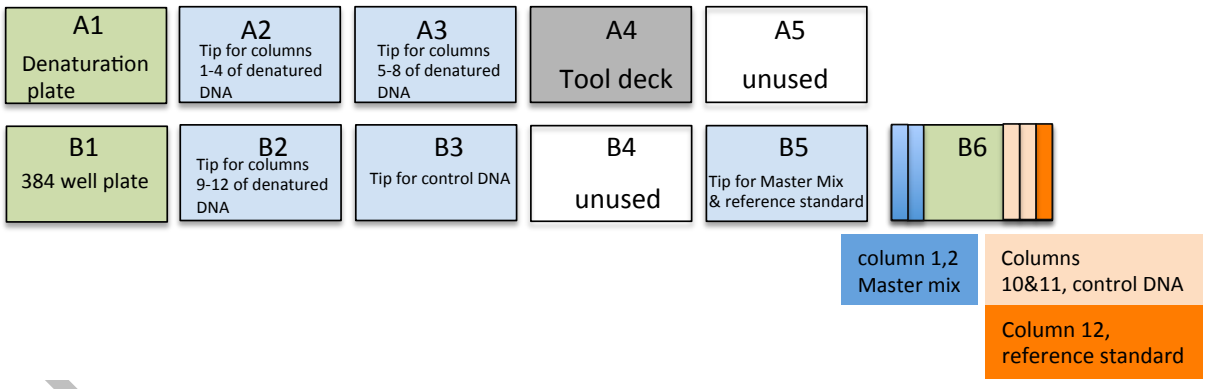
3.3.8. Use a new tip, pipette 25 µl of the content of DS5 into DS6. Pipette up and down 8-10 times.

NOTE: Steps *3.3.4-3.3.8* complete the serial dilutions of reference DNA. DS7 serves as the non-template control and last tube is empty.

3.3.9. Put the 8-strip tube with reference DNA (DS1-DS7) to the very right column of the Peltier block on your right side. DS1 should be on the upper right corner. Put the two control DNA strips next to the reference DNA. C1 and C9 should be on top.

3.4. Prepare PCR mixes.

Figure 1: Biomek 3000 layout for T and S runs



3.4.1. In a 5 ml tube, mix the following to make Complete T Master Mix

U (universal) Mix

T mix

10XSYBR green, fresh each day 5ul 10X SybrGreen

Platinum Taq polymerase

Reference the volume for each component using the Table taped on the Biomek 3000 (also at the end of this SOP)

Invert 10-15 times to mix, leave on ice.

3.4.2. Label a 384-well plate and put it on the 384 metal block. Make sure the well A1 is in the left upper corner.

3.4.3. Go to Biomek Software icon on desktop, open the program called "START HERE". Click the green arrow, you will be prompted to enter the number of column of samples. Afterwards, you will be prompted to put 1 or 2 strips of 8-strip 0.2 ml PCR tubes on the Peltier block that is on your upper right corner.
3.4.4. Add the amount of T run complete mix based on the prompt screen on Biomek software.
3.4.5. Put the Biomek p20 tip boxes on the Biomek decks as indicted by the computer screen.
3.4.6. Start Biomek program.
3.4.7. After Biomek finishes pipetting, seal the 384 well plate. Mix the samples on MixMate (Eppendorf) at 384 well "PCR384" setting. Spin down the plate in Sorval table top centrifuge (4 degree, 2000 rpm, 2 minutes). Place the plate on metal block on ice, cover with aluminum foil until ready to run.
3.4.8. Start the PCR program in Roche LC480.
For S run,
Repeat steps 3.4. 1 to 3.4.8 except use S run mix instead of T run mix.

APPENDIX A: Thermal cycling profile

Cycling for T PCR:
program 1:
94°C x 1 min. (Denaturation of DNA and activation of the hot start Taq DNA polymerase).
program 2:
Denature at 96°C for 1 second, anneal/extend at 54°C for 60 seconds, with fluorescence data collection (SINGLE), **30 cycles**.
program 3:
Melted at 95°C for 1 min., annealed at 54°C for 30 seconds, 95°C for 30 seconds with fluorescence data collection (CONTINOUS) during the rise to 95°C (Dissociation/Melting Curve).

Cycling for S PCR:
Program 1:
94°C for 1 min. (Denaturation of DNA and activation of the hot start Taq DNA polymerase).
Program 2:
Denature at 95°C for 15 seconds, anneal at 58°C for 1 second, extend at 72°C for 20 seconds, **8 cycles** (no data collection)
program 3:
Denature at 96°C for 1 second, anneal at 58°C for 1 second, extend at 72°C for 20 seconds, hold at 83°C for 7 seconds with fluorescence data collection (SINGLE), **34 cycles**.
program 4:
Melted at 95°C for 1 min., annealed at 54°C for 30 seconds, 95°C for 30 seconds with fluorescence data collection (CONTINOUS) during the rise to 95°C (Dissociation/Melting Curve

APPENDIX B: Volume for each component to make complete T master mix and complete S master mix

NOTE: all volumes in ul												
# of samples	8	16	24	32	40	48	56	64	72	80	88	96
# of columns	1	2	3	4	5	6	7	8	9	10	11	12
U mix	630	756	882	1008	1134	1260	1386	1512	1638	1764	1890	2016
T or S mix	291	349	407	465	523	581	639	697	755	813	872	930
1XSYBR	58	70	81	93	105	116	128	139	151	163	174	186
Platinum Taq	11.6	14.0	16.3	18.6	20.9	23.3	25.6	27.9	30.2	32.6	34.9	37.2
total volume	990	1188	1386	1584	1782	1980	2178	2376	2574	2772	2970	3169
each tube	119	144	169	194	218	243	268	293	317	171	183	196
# of strips	1	1	1	1	1	1	1	1	1	2	2	2

REVISION HISTORY

DATE	AUTHOR	REVISION #	REVISION REASON
July 31, 2009	Jue Lin	A	Initial Release
Jan 7, 2020	Jue Lin	F	Reformatted for TRN website

References:

1. Lin, J., et al., *Analyses and comparisons of telomerase activity and telomere length in human T and B cells: insights for epidemiology of telomere maintenance.* J Immunol Methods, 2010. **352**(1-2): p. 71-80.
2. Cawthon, R.M., *Telomere measurement by quantitative PCR.* Nucleic Acids Res, 2002. **30**(10): p. e47.