

MMQ-PCR TELOMERE ASSAY PROTOCOL- 2/5/14**MMQ-PCR Master Mix – Original Protocol, with Dec2013 alterations**Final Concentration of Reagents

.9 μ M telg
 .9 μ M telc
 .6 μ M albd2
 .6 μ M albu2
 .75X Sybr Green I
 1X Gene Amp Buffer II
 .8 mM dNTPs
 10 mM MgCl₂
 3 mM DTT
 1 M Betaine
 2.5 U AmpliTaq GOLD Polymerase
 PCR Grade H₂O to 15 μ L (for a 25 μ L rxn)

<u>1X Rxn Mix</u>	<u>¼ Plate (32 rxns Mix)</u>	<u>½ Plate (64 Rxn Mix)</u>	<u>Full Plate (128 Rxn Mix)</u>	<u>Reagent</u>
0.05625 μ L	1.8 μ L	3.6 μ L	7.2 μ L	100 μ M telg
0.05625 μ L	1.8 μ L	3.6 μ L	7.2 μ L	100 μ M telc
0.0375 μ L	1.2 μ L	2.4 μ L	4.8 μ L	100 μ M albd2
0.0375 μ L	1.2 μ L	2.4 μ L	4.8 μ L	100 μ M albu2
0.1875 μ L	6.0 μ L	12.0 μ L	24.0 μ L	100 X Sybr Green I (diluted in 1X Gene Amp Buffer II)
2.5 μ L	80 μ L	160 μ L	320 μ L	10 X GeneAmp Buffer II
0.8 μ L	25.6 μ L	51.2 μ L	102.4 μ L	25 mM dNTPs
0.25 μ L	8.0 μ L	16.0 μ L	32.0 μ L	1M MgCl ₂
0.75 μ L	24.0 μ L	48.0 μ L	96.0 μ L	100 mM DTT
5.0 μ L	160.0 μ L	320.0 μ L	640.0 μ L	5 M Betaine
0.5 μ L	16.0 μ L	32.0 μ L	64.0 μ L	5 U/ μ L AmpliTaq GOLD
<u>4.2625 μL</u>	<u>154.4 μL</u>	<u>308.8 μL</u>	<u>617.6 μL</u>	PCR Grade H ₂ O
15 μ L	480 μ L	960 μ L	1920 μ L	

Stock concentration of Reagents Aliquot Size

Primer pellets are resuspended in 100 μ L 1X TE (made with PCR Grade H₂O), then diluted to 100 μ M in PCR Grade H₂O. Any other required dilutions are in PCR grade H₂O.

100 μ M telg	33 μ L	
100 μ M telc	33 μ L	
100 μ M albd2	23 μ L	
100 μ M albu2	23 μ L	
10000X Sybr Green I	5 μ L	
1X Gene Amp Buffer II	99 μ L	<i>(add 1 μL 10000X Sybr Green I to 99 μL 1X Gene Amp Buffer II to make 100X Sybr Green I; this dilution can be used and stored at -20C for up to 1 week)</i>
10X Gene Amp Buffer II	350 μ L	
25 mM dNTPs	110 μ L	
1 M MgCl ₂ (RNA/PCR Grade)	35 μ L	
100 mM DTT	100 μ L	
5 M Betaine	700 μ L	
PCR Grade H ₂ O	13 mL & 900 μ L	

Preparing Aliquots for MMQ-PCR Master Mix

Remember: label all aliquot tubes with the name of the reagent and the date of aliquot (month/year)

- **1X TE**
 - First, make 1X TE as follows:
 - In a 15mL tube, add: [**Fisher Cat# 352096**]
 - 100µl 1M Tris-Cl pH 7.5 [**Fisher Cat# 1757-100**]
 - 20µl 0.5M EDTA pH 8.0 [**Fisher Cat# PR-V4231**]
 - Then, make volume up to 10mL (1000µl) with PCR Grade H₂O (i.e. add 880µl PCR Grade H₂O) [**Life Technologies Cat# AM9937**]
 - Mix well
 - Filter sterilize as follows:
 - Pour mix into a new PCR loading tray (white plastic tray) [**Fisher Cat# 89094-662**]
 - Suck entire mix into a new plastic syringe [**Fisher Cat# 148232A**]
 - Attach new filter to end of syringe [**Fisher Cat# 28145-477**]
 - In a NEW 15mL tube, push syringe plunger and drip mix into the new tube
 - (Do not re-use syringe, filter or loading tray)
 - 1.5mL tubes labeled "1X TE" with date (month/year) [**USA Scientific Cat# 1615-5599**]
 - Aliquot sterilized mix into 1.5µl tubes (~1.4 µl)
 - Store at room temperature (RT) (PCR room telomere reagent cabinet in box labeled "1XTE for Telomere Assay")
 - This will be used to dilute/rehydrate oligos (see below).

- **Oligos (telg, telc, albd2 and albu2) [See "Sigma Oligo ordering protocol"]**
 - Check individual oligo tube for nmol concentration ([nmol]=α)
 - Observe (if possible) where the dehydrated oligo is in the tube (so that you don't miss it when you add 1XTE)
 - Rehydrate oligo with:
 - 100µl 1XTE (created in protocol above)
 - Mix well
 - Dilute rehydrated oligo to 100µm in the following manner:
 - Add (10α) µl -100 µl PCR Grade H₂O
(e.g. if the nmol concentration on the tube reads 24.6nmol, then add 146µl PCR Grade H₂O to the oligo already rehydrated in the 100µl 1XTE)
 - 0.5mL tubes labeled "telg", "telc", "albd2" or "albu2" with date (month/year) [**USA Scientific Cat# 1605-0099**]
 - For "telg" and "telc", aliquot 33µl volumes
 - For "albd2" and "albu2", aliquot 23µl volumes
 - Store at -20°C (PCR room freezer second top shelf in box labeled "Telomere Oligos")

- **SYBR Green [Life Technologies Cat# S7563]**
 - 0.5mL tubes labeled "SYBR" with date (month/year)
 - Aliquot 5µl volumes
 - Store at -20°C (PCR room freezer second top shelf in box labeled "Telomere Reagents")
 - These aliquots are made to prevent multiple freeze-thaw cycles and protect from contamination – this is NOT the final concentration that you will use in your plates (see "1X Gene Amp Buffer II, below)

- **1X Gene Amp Buffer II [Life Technologies comes with Amplitaq GOLD Cat# N8080249]**
 - 0.5mL tubes labeled "1X Gene Buff II" with date (month/year)
 - To each tube, add:
 - 89.1µl PCR Grade H₂O
 - 9.9 µl 10X Gene Amp Buffer II

MMQ-PCR (Page 3 of 13)

- This makes 99µl aliquots of 1X Buffer
- Store at -20°C (PCR room freezer second top shelf in box labeled “Telomere Reagents”)
- Before use, add:
 - 1µl SYBR Green (Note: this creates a 100µl aliquot of 100X SYBR – i.e. enough for 4 plates.)
- **10X Gene Amp Buffer II [Life Technologies comes with Amplitaq GOLD Cat# N8080249]**
 - 0.5mL tubes labeled “10X Gene Buff II” with date (month/year)
 - Aliquot 35µl volumes
 - Store at -20°C (PCR room freezer second top shelf in box labeled “Telomere Reagents”)
- **5M Betaine [Fisher Cat# 77507-10mL]**
 - 1.5mL tubes labeled “Betaine” with date (month/year)
 - Aliquot 700 µl volumes
 - Store at -20°C (PCR room freezer second top shelf in box labeled “Telomere Reagents”)
- **1M MgCl₂ [Life Technologies Cat# AM9530G or Fisher Cat# 50-152-107]**
 - 0.5mL tubes labeled “MgCl₂” with date (month/year)
 - Aliquot 35µl volumes
 - Store at +4°C (Monkey room fridge door in box labeled “MgCl₂ for Telomere Assay”)
- **dNTPs [NEB Cat# N0446S]**
 - In a 1.5mL tube, mix:
 - equal parts of 100mM dGTP, dCTP, dATP and dTTP stocks (e.g. 225µl of each)
 - 0.5mL tubes labeled “dNTPs” with date (month/year)
 - Aliquot 110 µl volumes from the created mix
 - Store at -20°C (PCR room freezer second top shelf in box labeled “Telomere Reagents”)
- **100mM DTT [RPI Corp Cat# D11000-5.0]**
 - First, make 10mL 0.01M NaOAc pH 5.2 as follows:
 - In a 15mL tube, add:
 - 33.33 µl 3M NaOAc pH 5.2 [Fisher Cat# BM-225]
 - 99.37µl PCR Grade H₂O
 - Mix well
 - Second, add:
 - 0.1545g DTT (use a scale with accuracy to at least 3 decimal places)
 - Mix well
 - Filter sterilize as follows:
 - Pour mix into a new PCR loading tray (white plastic tray)
 - Suck entire mix into a new plastic syringe
 - Attach new filter to end of syringe
 - In a NEW 15mL tube, push syringe plunger and drip mix into the new tube.
 - (Do not re-use syringe, filter or loading tray)
 - 0.5mL tubes labeled “DTT” with date (month/year)
 - Aliquot 100 µl volumes from the created mix
 - Store at -20°C (PCR room freezer second top shelf in box labeled “Telomere Reagents”)
 - **NOTE:** It is not necessary, nor practical, to aliquot the entire 10mL in one go. Aliquot a reasonable amount (e.g. 20 tubes) and then store the remaining mix, in the final 15mL tube with a clear label “DTT” with date (month/year), at -20°C (PCR room freezer second top shelf in box with incoming reagents). This can be thawed and used to make additional aliquots at a later time point.

Preparing Standards

(NB: Standard biological sample type must match sample type of entire plate)

- 1) Obtain multiple tubes/swabs/spot cards of the relevant biological sample source from Stacy (SD). Note: the amount you can feasibly extract at one time will vary by sample type (i.e. peripheral blood has higher yield than buccal). Extract a large amount of the relevant biological sample type (see relevant laboratory protocol for extraction procedure), which can then be used for many future telomere plates (eg: 15mL @ 30ng/ μ L). Use multiple extractions (e.g. >10 buccal swabs) and then combine them together to get the largest possible volume at best possible concentration. The goal is to be able to use this same final mixed sample volume for as many plates as possible (to avoid possible variation between runs/studies), however the total amount you can extract at one time will be limited by practicalities (e.g. impractical to extract 100 swabs at once....).
- 2) Determine the concentration of intact dsDNA in the total volume by Qubit Assay (ensure sample is fully defrosted and well mixed before reading is made). Record this value in a safe place (e.g. lab book) as this will be important for all future aliquots of that specific standard SD-DNA that you make from this bulk extraction. You should have a known Qubit concentration recorded for each of the different biological sample sources that you have made large stocks of for future study (e.g. peripheral blood, saliva, buccal, blood spot etc.).

[Life Technologies Cat# Q32853 (Qubit Kit) and Cat# Q32856 (Qubit Tubes)]

WARNING: Put on a fresh pair of gloves, THEN put on lab coat from sterile bag BEFORE entering the hood. Change gloves immediately before putting on lab coat from sterile bag, or removing lab coat to return it to the sterile bag. If you leave the hood for ANY reason (even just to go to the -20 PCR freezer), then change gloves immediately before re-entering hood. If you leave the room for ANY reason, then change gloves immediately BEFORE removing coat, and return coat to sterile bag. When you return to room, put on new gloves and THEN lab coat immediately before re-entering hood. Regularly have your coat dry cleaned (by Stacy), e.g. monthly, and keep additional coats (as soon as they return) in sterile bags ready for use (rotate coats so you don't end up without one).

- 3) Aliquot large stock sample into 1.5mL tubes labeled on top with "sample type", "assay intended for", and the "tube number" for ease of tracking (e.g. "SD BUCCAL TELOMERE 1 of 5"). Label the side of tube with the Qubit reading for that sample and the date the tube was created (month/day/year). Store tubes in a single stock standard SD-DNA box (ORANGE) that is reserved for telomere reagents only. Store in -20 PCR freezer, on telomere shelf (2nd from top) until required for aliquot dilutions.
- 4) Create a set of aliquot dilutions for individual standard curves as required. First, calculate the amount of stock SD-DNA required to add to H₂O to create a total volume of 1.5mL at 2ng/ μ L (e.g. if Qubit is 30ng/ μ L, then take 100 μ L of that stock and mix with 1400 μ L of PCR grade H₂O in a 1.5mL tube. These calculations can easily be made with the online dilution calculator at <http://www.endmemo.com/bio/dilution.php>). Label the top of 20x 0.5mL tubes with "sample type" ("SD-E" for buccal, "SD-B" for blood, SD-S" for saliva, SD-BS" for blood spot) and dilution (i.e. 2ng/ μ L). Label the side of the tube with the date created (month/year). Aliquot 75 μ L of the diluted (2ng/ μ L) standard into each tube and store in single diluted standard SD-DNA box (BLUE) that is reserved for telomere diluted standards only. Store in -20 PCR freezer, on telomere shelf (2nd from top) until required for plate set up.
- 5) To create a 2 fold serial dilution standard for a single plate set up: take a single aliquot tube from the freezer, fully defrost, pipette up and down to mix sample. Obtain a PCR strip **[Fisher Cat# 21-402-169]** and place in first column of PCR strip holder. Prepare a 2 fold serial dilution (in PCR Grade H₂O) for the standard curve, beginning with 20 ng/rxn (10 μ L 2 ng/ μ L template).
 - a. Add 35 μ L PCR Grade H₂O to tubes B-H in PCR Tube Strip 1.
 - b. Add 35 μ L Standard Curve Full Strength dilution to tube A in PCR Strip 1.
 - c. Add 35 μ L Standard Curve Full Strength to Tube B and mix with H₂O by pipetting up and down.
 - d. Add 35 μ L of tube B to tube C and mix with H₂O by pipetting up and down; repeat in each tube until G.
 - e. Leave H as H₂O ONLY as this is the No Template Control (NTC).

*Note: Therefore, every well of this strip will have 35 μ L in it, except well G, which will have 70 μ L.

MMQ-PCR (Page 5 of 13)

WARNING: Each time you are finished using the airclean hood, wipe down pipettes and inside of hood before leaving. Check you have enough tips/tubes/plates/wells inside the hood for the next use. Ensure all biological samples and reagents are removed and stored in appropriate freezer/fridge (DO NOT turn on UV light if samples/reagents are present). Close airclean door and set UV light to run for 15mins (just press lightbulb button and timer should start counting down, as long as door is closed). Change gloves immediately BEFORE removing lab coat, and return coat to sterile bag until next use.

Preparing individual samples

- 1) Samples extracted using relevant laboratory protocol.
- 2) Determine the concentration of intact dsDNA by Qubit Assay. Data should be recorded (and double checked) in data computer.
- 3) Store samples in 1.5mL tubes in appropriate order in individual styrofoam cohort boxes in -20 PCR freezer until required for telomere assays. See laboratory labeling protocol for instructions on labeling style required and color stickers used for each specific cohort/sample type.
- 4) Obtain Qubit data from data computer and make a list in excel with Qubit reading and sample name for the samples you intend to run (only do this for a single sample type at a time as they must be run on plate with same biological sample standard). Sort the file by Qubit reading (as this will make the plate set up easier) and print list with 24 samples per page (as this is how many samples fit on a single plate).
- 5) Take printed list to PCR room, put on a new pair of gloves, and find the appropriate sample tubes. Ensure you have obtained the correct sample type for each individual. Place the 24 tubes in 3 columns of 8 in a tube rack, in the order intended for the plate, ready to create assay dilutions in 8-tube PCR strips.

WARNING: Put on a fresh pair of gloves, THEN put on lab coat from sterile bag BEFORE entering the hood. Change gloves immediately before putting on lab coat from sterile bag, or removing lab coat to return it to the sterile bag. If you leave the hood for ANY reason (even just to go to the -20 PCR freezer), then change gloves immediately before re-entering hood. If you leave the room for ANY reason, then change gloves immediately BEFORE removing coat, and return coat to sterile bag. When you return to room, put on new gloves and THEN lab coat immediately before re-entering hood. Regularly have your coat dry cleaned (by Stacy), e.g. monthly, and keep additional coats (as soon as they return) in sterile bags ready for use (rotate coats so you don't end up without one).

- 6) Obtain 3 PCR strips [**Fisher Cat# 21-402-105**] with lids and label the top end of the lid strip according to next numerical order following on from previous plate in lab book (e.g. 100, 101, 102). Note the number and color of the PCR strip in lab book for future reference. Label the left side of the tubes with the sample IDs, include a letter following the ID to represent the sample type if the cohort has multiple samples from the same individual. Prepare samples by adding dilutions to PCR tube strips according to planned plate positions following the rules below (to end up with 75 μ l volumes in each well of strips).
 - a. If Qubit(x) is >4ng/ μ l add 1.5 μ l sample to 73.5 μ l PCR grade H₂O.
 - b. If 3<x<4ng/ μ l add 2 μ l sample to 73 μ l PCR grade H₂O.
 - c. If 2<x<3ng/ μ l add 3 μ l sample to 72 μ l PCR grade H₂O.
 - d. If 1<x<2ng/ μ l add 4 μ l sample to 71 μ l PCR grade H₂O.
 - e. If Qubit(x) is <1ng/ μ l add 5 μ l sample to 70 μ l PCR grade H₂O.

*Note: if you are having trouble with a sample type having super late take offs, you may want to "push" the dilutions (i.e. add 1 μ l more of each sample, and 1 μ l less of H₂O for each tube.... Same total volume of 75 μ l)

*Note: this is enough to run both P1 and P2 (see instructions for "assembling the reaction plate") from these PCR strips. Store the strips in the -20 PCR freezer between runs, and move to long term stored PCR strip boxes (at back of -20 PCR freezer, 2nd top shelf) once both plates are successfully run. If one plate fails entirely, you can make 2nd dilutions within the same PCR strip.

WARNING: Each time you are finished using the airclean hood, wipe down pipettes and inside of hood before leaving. Check you have enough tips/tubes/plates/wells inside the hood for the next use. Ensure all biological samples and reagents are removed

MMQ-PCR (Page 6 of 13)

and stored in appropriate freezer/fridge (DO NOT turn on UV light if samples/reagents are present). Close airclean door and set UV light to run for 15mins (just press lightbulb button and timer should start counting down, as long as door is closed). Change gloves immediately BEFORE removing lab coat, and return coat to sterile bag until next use.

Assembling the Reaction Plate

Before you start in the hood...

- 1) In your lab book, list the type of assay you are about to perform (i.e. "Telomere"), the cohort (e.g. BEIP), the sample type (e.g. buccal), and the date (month/day/year).
- 2) List any variations in the sample dilution rules (see step 7) you intend to use (if unchanged, write "dilⁿ as previous").
- 3) Create a diagram of the plate set up that you intend to run (this is a lot easier if your lab book has grid lines). Remember to include the sample type for the standard (e.g. SD-B for blood), the color/numbers of the PCR strips you intend to use (see step 7), and the individual sample IDs. (Additional note: Include a letter to indicate sample type after the sample IDs if the cohort has multiple sample types) eg:

				<i>"Color strip 100"</i>			<i>"Color strip 101"</i>			<i>"Color strip 102"</i>		
	1	2	3	4	5	6	7	8	9	10	11	12
A	(Standard1) SD-"type"			<i>"Sample ID 1"</i>			<i>"Sample ID 9"</i>			<i>"Sample ID 17"</i>		
B	(S2)			<i>"Sample ID 2"</i>			<i>"Sample ID 10"</i>			<i>"Sample ID 18"</i>		
C	(S3)			<i>"Sample ID 3"</i>			<i>"Sample ID 11"</i>			<i>"Sample ID 19"</i>		
D	(S4)			<i>"Sample ID 4"</i>			<i>"Sample ID 12"</i>			<i>"Sample ID 20"</i>		
E	(S5)			<i>"Sample ID 5"</i>			<i>"Sample ID 13"</i>			<i>"Sample ID 21"</i>		
F	(S6)			<i>"Sample ID 6"</i>			<i>"Sample ID 14"</i>			<i>"Sample ID 22"</i>		
G	(S7)			<i>"Sample ID 7"</i>			<i>"Sample ID 15"</i>			<i>"Sample ID 23"</i>		
H	NTC			<i>"Sample ID 8"</i>			<i>"Sample ID 16"</i>			<i>"Sample ID 24"</i>		

- 4) As shown in your diagram, each sample is run in triplicate. Each plate is run TWO times, and each of these two runs will have the samples in DIFFERENT wells (to check there is no effect of well location, or inter-run differences). One run is conducted in the orientation shown in the diagram (P1), and the other is rotated 180° (P2). This means that, for P2, well A1 in the diagram is moved to H12, A2 is G12 and so on. This 2nd plate (P2) of the pair can be achieved by simply rotating your PCR strips 180° before you begin loading the samples to that particular plate (P2).

In the hood...

WARNING: Put on a fresh pair of gloves, THEN put on lab coat from sterile bag BEFORE entering the hood. Change gloves immediately before putting on lab coat from sterile bag, or removing lab coat to return it to the sterile bag. If you leave the hood for ANY reason (even just to go to the -20 PCR freezer), then change gloves immediately before re-entering hood. If you leave the room for ANY reason, then change gloves immediately BEFORE removing coat, and return coat to sterile bag. When you return to room, put on new gloves and THEN lab coat immediately before re-entering hood. Regularly have your coat dry cleaned (by Stacy), e.g. monthly, and keep additional coats (as soon as they return) in sterile bags ready for use (rotate coats so you don't end up without one).

- 5) Prepare PCR mix for the appropriate number of wells according to the chart on the previous page. Be sure to choose a tube volume that allows room for the solution to mix when vortexing (full plate mix fits in 5 mL tube [Fisher Cat# 03-391-162] etc.). It is better to only run full plates as this helps with establishing plate reliability, minimizing plate variations, is more cost effective, and saves time both for plate set up and also analysis. WRITE the volumes you use in your lab book BEFORE you begin, and then tick them off as you add them to the mix. Do this for both P1 and P2. Eg:

		<i>"Month/day/year"</i>	<i>"Month/day/year"</i>
		TEL201 (P1)	TEL202 (P2)
100µm telg	7.2	✓	✓
100µm telc	7.2	✓	✓
100µm albd2	4.8	✓	✓

MMQ-PCR (Page 7 of 13)

100µm albu2	4.8	✓	✓
100X Sybr Green (in 1X GeneBuffer)	24.0	✓	✓
10X Gene Buffer	320.0	✓	✓
25mM dNTPs	102.4	✓	✓
1M MgCl ₂	32.0	✓	✓
100mM DTT	96.0	✓	✓
5M Betaine	640.0	✓	✓
5U/µl AmplitaqGOLD	64.0	✓	✓
PCR Grade H ₂ O	617.6	✓	✓
TOTAL VOLUME	1920µl		

- 6) Prepare a 2 fold serial dilution (in PCR Grade H₂O) for the standard curve (this is also described in “preparing the standard section”)
- Add 35 µl PCR Grade H₂O to tubes B-H in PCR Tube Strip 1.
 - Add 35 µl Standard Curve Full Strength dilution to tube A in PCR Strip 1.
 - Add 35 µl Standard Curve Full Strength to Tube B and mix with H₂O by pipetting up and down.
 - Add 35 µl of tube B to tube C and mix with H₂O by pipetting up and down; repeat in each tube until G.
 - Leave H as H₂O only for the No Template Control (NTC).
- 7) Prepare the samples curve (this is also described in “preparing the samples section”). Obtain 3 PCR strips with lids and label the top end of the lid strip according to next numerical order following on from previous plate in lab book (e.g. 100, 101, 102). As shown above, note the number and color of the PCR strip in lab book for future reference. Label the left side of the tubes with the sample IDs, include a letter following the ID to represent the sample type if the cohort has multiple samples from the same individual. Prepare samples by adding dilutions to PCR tube strips according to planned plate positions following the rules below (to end up with 75µl volumes in each well of strips).
- If Qubit(x) is >4ng/µl add 1.5µl sample to 73.5µl PCR grade H₂O.
 - If 3<x<4ng/µl add 2µl sample to 73µl PCR grade H₂O.
 - If 3<x<3ng/µl add 3µl sample to 72µl PCR grade H₂O.
 - If 3<x<2ng/µl add 4µl sample to 71µl PCR grade H₂O.
 - If Qubit(x) is <1ng/µl add 5µl sample to 70µl PCR grade H₂O.
- *Note: if you are having trouble with a sample type having super late take offs, you may want to “push” the dilutions (i.e. add 1µl more of each sample, and 1µl less of H₂O for each tube.... Same total volume of 75µl)
- *Note: this is enough to run both P1 and P2 from these PCR strips. Store the strips in the -20 PCR freezer between runs, and move to long term stored PCR strip boxes (at back of -20 PCR freezer, 2nd top shelf) once both plates are successfully run. If one plate fails entirely, you can make 2nd dilutions within the same PCR strip.
- 8) Vortex PCR Mix and:
- For a full plate, pour the vortexed PCR mix into a multichannel trough [**Fisher Cat# 89094-6664**].
 - For a ½ plate or less, pipet the appropriate amount of PCR mix into each well of a PCR Tube Strip [**Fisher Cat# 21-402-105**] (4 rxns volume for each triplicate).
 - Pipet 15 µL into each rxn well of 96 well PCR plate [**BioRad Cat# HSP9601**].
- 9) Add 10 µL of each template sample to the appropriate rxn well and mix by pipetting up and down.
- 10) Seal the plate with Optically Clear Film [**BioRad Cat# MSB-1001**].
- 11) Centrifuge the plate 2 min.
- 12) Insert plate into BioRad CFX96 machine. Ensure the plate is up the correct way. Check plate is sealed correctly (use a kimwipe [**Fisher Cat# 34155 (small) and Cat# 34256 (large)**] to go firmly around the edges one last time.
- 13) From the attached computer, run PCR Protocol below. This protocol should ideally be saved to your MMQ-PCR folder and you just need to select the correct run file each time. When you click “run” you will be asked to label your plate BEFORE the run will begin. Label plate with “TEL” and number that follows your previous plate (e.g. TEL207) the cohort (e.g. BEIP), sample type (e.g. “Buccal”), the date (month/day/year) and whether the plate is P1 or P2 of the plate pair.

MMQ-PCR (Page 8 of 13)

- 14) Store the PCR strips containing your sample dilutions in the -20 PCR freezer (either ready for you P2 run, or in long term storage in case they are required at a later time point).

WARNING: Each time you are finished using the airclean hood, wipe down pipettes and inside of hood before leaving. Check you have enough tips/tubes/plates/wells inside the hood for the next use. Ensure all biological samples and reagents are removed and stored in appropriate freezer/fridge (DO NOT turn on UV light if samples/reagents are present). Close airclean door and set UV light to run for 15mins (just press lightbulb button and timer should start counting down, as long as door is closed). Change gloves immediately BEFORE removing lab coat, and return coat to sterile bag until next use.

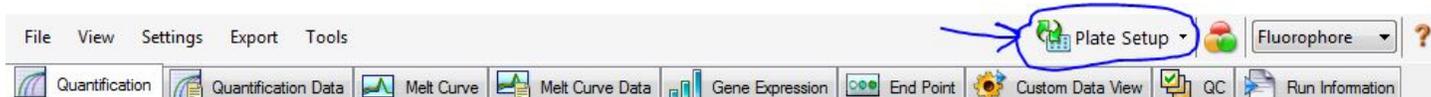
PCR Protocol

1. 95C 15:00
2. 94C 00:15
3. 49C 01:00
4. Goto 2 1X
5. 94C 00:15
6. 59C 00:30
7. Goto 5 3X
8. 85C 00:15
9. 73.5C 00:30 *Signal Acquisition
10. Goto 8 19X
11. 94C 00:15
12. 84C 00:30 *Signal Acquisition
13. Goto 11 29X
14. Melt curve 59C-95C Increment .5C for 00:05 *Signal Acquisition

Data Quality Control

AT THE CFX96...

- 1) Go into the "plate set up" feature (button at top right if run is completed) to tell the CFX where you standard is.



- a. Highlight all wells and click "select Fluorophores" (right hand side).
- b. Check the box next to "SYBR" and uncheck all other boxes. Click "OK".
- c. While all wells are still highlighted, under the word "load" (right hand side, note that there are multiple places that say "load" but only one will have the "SYBR" option), check the box next to "SYBR". Now all the wells should have "SYBR" written on them.
- d. Next, highlight only your 3 NTC wells and select "NTC" from the sample type menu on the right (those wells should turn yellow and be called NTC).
- e. Highlight the 3x7 wells of the standard and select "standard" from the sample type menu (they should turn green)
- f. While the standard is still highlighted click "replicate series". Select "3" from the "replicate size" drop down menu. Select "horizontal". Click "Apply" (those wells should now be labeled in sets of 3 from Std-1 though Std7).
- g. While the standard is still highlighted click "dilution series". In the "dilution factor" field, type "2". Then, what you enter will differ between P1 and P2 plates:
 - i. If it is a P1 plate: enter 2E-3 in the "starting concentration" field, and check the box for "decreasing". Click "Apply". Your standard should now have concentration values written in each well (highest 2.00E-03 wells A1-3 through to lowest 3.13E-05 wells G1-3)

MMQ-PCR (Page 9 of 13)

- ii. If it is a P2 plate: enter 3.13E-5 in the “starting concentration” field, and check the box for “increasing”. Click “Apply”. (lowest 3.13E-05 wells B10-12 through to highest 2.00E-03 wells H10-12)
*Note: the actual concentrations are not relevant to the calculation the CFX performs to calculate efficiency. The most relevant part is the dilution factor. If you don’t type “2” and select the appropriate “increasing” or “decreasing” box, your standard curve will look wrong.
- h. Highlight the 9x8 wells of the unknown samples and select “unknown” from the sample type menu (they should turn blue).
- i. While the “unknown” section is still highlighted click “replicate series”. Select “3” from the “replicate size” drop down menu. Select “horizontal”. Click “Apply” (those wells should now be labeled in sets of 3 from Unk-1 though Unk-24). [NB: this will appear in the same orientation regardless of whether the plate is a P1 or P2. Do not be misled by the numbering as it is not consistent and should only be used to keep track of triplicates during QC analysis of individual plates.]

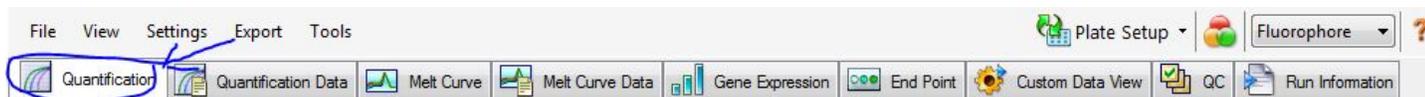
P1 set up looks like this:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std-1 2.00E-03	Std-1 2.00E-03	Std-1 2.00E-03	Unk-1 SYBR	Unk-1 SYBR	Unk-1 SYBR	Unk-2 SYBR	Unk-2 SYBR	Unk-2 SYBR	Unk-3 SYBR	Unk-3 SYBR	Unk-3 SYBR
B	Std-2 1.00E-03	Std-2 1.00E-03	Std-2 1.00E-03	Unk-4 SYBR	Unk-4 SYBR	Unk-4 SYBR	Unk-5 SYBR	Unk-5 SYBR	Unk-5 SYBR	Unk-6 SYBR	Unk-6 SYBR	Unk-6 SYBR
C	Std-3 5.00E-04	Std-3 5.00E-04	Std-3 5.00E-04	Unk-7 SYBR	Unk-7 SYBR	Unk-7 SYBR	Unk-8 SYBR	Unk-8 SYBR	Unk-8 SYBR	Unk-9 SYBR	Unk-9 SYBR	Unk-9 SYBR
D	Std-4 2.50E-04	Std-4 2.50E-04	Std-4 2.50E-04	Unk-10 SYBR	Unk-10 SYBR	Unk-10 SYBR	Unk-11 SYBR	Unk-11 SYBR	Unk-11 SYBR	Unk-12 SYBR	Unk-12 SYBR	Unk-12 SYBR
E	Std-5 1.25E-04	Std-5 1.25E-04	Std-5 1.25E-04	Unk-13 SYBR	Unk-13 SYBR	Unk-13 SYBR	Unk-14 SYBR	Unk-14 SYBR	Unk-14 SYBR	Unk-15 SYBR	Unk-15 SYBR	Unk-15 SYBR
F	Std-6 6.25E-05	Std-6 6.25E-05	Std-6 6.25E-05	Unk-16 SYBR	Unk-16 SYBR	Unk-16 SYBR	Unk-17 SYBR	Unk-17 SYBR	Unk-17 SYBR	Unk-18 SYBR	Unk-18 SYBR	Unk-18 SYBR
G	Std-7 3.13E-05	Std-7 3.13E-05	Std-7 3.13E-05	Unk-19 SYBR	Unk-19 SYBR	Unk-19 SYBR	Unk-20 SYBR	Unk-20 SYBR	Unk-20 SYBR	Unk-21 SYBR	Unk-21 SYBR	Unk-21 SYBR
H	NTC SYBR	NTC SYBR	NTC SYBR	Unk-22 SYBR	Unk-22 SYBR	Unk-22 SYBR	Unk-23 SYBR	Unk-23 SYBR	Unk-23 SYBR	Unk-24 SYBR	Unk-24 SYBR	Unk-24 SYBR

P2 set up looks like this:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk-1 SYBR	Unk-1 SYBR	Unk-1 SYBR	Unk-2 SYBR	Unk-2 SYBR	Unk-2 SYBR	Unk-3 SYBR	Unk-3 SYBR	Unk-3 SYBR	NTC SYBR	NTC SYBR	NTC SYBR
B	Unk-4 SYBR	Unk-4 SYBR	Unk-4 SYBR	Unk-5 SYBR	Unk-5 SYBR	Unk-5 SYBR	Unk-6 SYBR	Unk-6 SYBR	Unk-6 SYBR	Std-1 3.13E-05	Std-1 3.13E-05	Std-1 3.13E-05
C	Unk-7 SYBR	Unk-7 SYBR	Unk-7 SYBR	Unk-8 SYBR	Unk-8 SYBR	Unk-8 SYBR	Unk-9 SYBR	Unk-9 SYBR	Unk-9 SYBR	Std-2 6.26E-05	Std-2 6.26E-05	Std-2 6.26E-05
D	Unk-10 SYBR	Unk-10 SYBR	Unk-10 SYBR	Unk-11 SYBR	Unk-11 SYBR	Unk-11 SYBR	Unk-12 SYBR	Unk-12 SYBR	Unk-12 SYBR	Std-3 1.25E-04	Std-3 1.25E-04	Std-3 1.25E-04
E	Unk-13 SYBR	Unk-13 SYBR	Unk-13 SYBR	Unk-14 SYBR	Unk-14 SYBR	Unk-14 SYBR	Unk-15 SYBR	Unk-15 SYBR	Unk-15 SYBR	Std-4 2.50E-04	Std-4 2.50E-04	Std-4 2.50E-04
F	Unk-16 SYBR	Unk-16 SYBR	Unk-16 SYBR	Unk-17 SYBR	Unk-17 SYBR	Unk-17 SYBR	Unk-18 SYBR	Unk-18 SYBR	Unk-18 SYBR	Std-5 5.01E-04	Std-5 5.01E-04	Std-5 5.01E-04
G	Unk-19 SYBR	Unk-19 SYBR	Unk-19 SYBR	Unk-20 SYBR	Unk-20 SYBR	Unk-20 SYBR	Unk-21 SYBR	Unk-21 SYBR	Unk-21 SYBR	Std-6 1.00E-03	Std-6 1.00E-03	Std-6 1.00E-03
H	Unk-22 SYBR	Unk-22 SYBR	Unk-22 SYBR	Unk-23 SYBR	Unk-23 SYBR	Unk-23 SYBR	Unk-24 SYBR	Unk-24 SYBR	Unk-24 SYBR	Std-7 2.00E-03	Std-7 2.00E-03	Std-7 2.00E-03

- j. Click “OK” at the bottom right of the “plate editor” window.
- 2) Go to the “Quantitation” tab (first tab, top left). Check that your curves look “right” on both the “9” (TEL) and the “12” (SCG) Steps (switch between these using the drop menu on the middle right of screen). Curves should not have early plateaus, strange Ct humps, inverted amplification curves, etc.



- 3) Check that your PCR efficiencies on both the TEL and the SCG are between 90%-110% with corresponding high R² values.
- 4) Check that your PCR efficiencies on both the TEL and the SCG do not differ from each other by more than 10% (92.4% SCG and 98.2% TEL is ok whereas 92.4% SCG and 108.3% TEL is not)

If all these criteria are met for P1- YAY- go run your P2 plate.

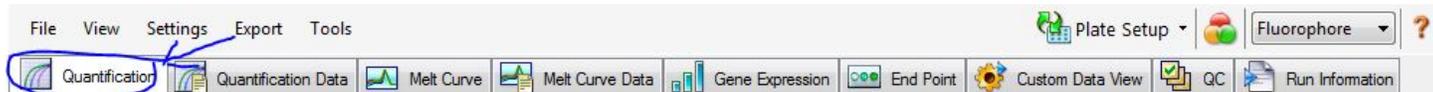
If all these criteria are met for P1 AND P2- double YAY- go do the paired plate analysis and generate some sweet, sweet data.

If these criteria are NOT met, your plate needs to be repeated (do not pass go, do not collect \$200). Make sure you THINK about whether there are any apparent reasons that it might have failed before you go ahead and repeat the plate. Sometimes there isn't an obvious reason, and it is just the gods of science being mean, but if you have the same issue on consecutive plates, you may have a serious assay issue that needs to be addressed before you can continue.

If you have been successful in running you plates and passing the above QC steps, copy the BioRad files for P1 and P2 to a thumb drive and return to your computer. You will need to have both BioRad and Microsoft Excel software installed to complete the next steps in QC and data analysis.

ON YOUR COMPUTER...

- 1) Find your P1 and P2 BioRad files on your thumb drive and save them to your data folder. Open both files.
- 2) Find and open the Excel file template "Zoe's Awesome TS Ratios Template". BEFORE making any edits to this template, SAVE AS an appropriate NEW FILE NAME in your data folder (e.g. "TEL101 and TEL102 - BEIP TP4").
- 3) USING THE P1 AND P2 BIORAD FILE:
 - a. Check that the NTC:
 - i. Go to the "Quantitation" tab (first tab, top left).



- ii. On the well set up image (bottom left), unselect the unknown samples (click and drag to highlight them in red, once you finish the selection, they will turn grey). Now, only your 3x7 wells of standard should remain blue (active).

P1 well set up image for **standard** analysis:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std1	Std1	Std1	Unk1	Unk1	Unk1	Unk2	Unk2	Unk2	Unk3	Unk3	Unk3
B	Std2	Std2	Std2	Unk4	Unk4	Unk4	Unk5	Unk5	Unk5	Unk6	Unk6	Unk6
C	Std3	Std3	Std3	Unk7	Unk7	Unk7	Unk8	Unk8	Unk8	Unk9	Unk9	Unk9
D	Std4	Std4	Std4	Unk10	Unk10	Unk10	Unk11	Unk11	Unk11	Unk12	Unk12	Unk12
E	Std5	Std5	Std5	Unk13	Unk13	Unk13	Unk14	Unk14	Unk14	Unk15	Unk15	Unk15
F	Std6	Std6	Std6	Unk16	Unk16	Unk16	Unk17	Unk17	Unk17	Unk18	Unk18	Unk18
G	Std7	Std7	Std7	Unk19	Unk19	Unk19	Unk20	Unk20	Unk20	Unk21	Unk21	Unk21
H	NTC	NTC	NTC	Unk22	Unk22	Unk22	Unk23	Unk23	Unk23	Unk24	Unk24	Unk24

P2 well set up image for **standard** analysis:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk1	Unk1	Unk1	Unk2	Unk2	Unk2	Unk3	Unk3	Unk3	NTC	NTC	NTC
B	Unk4	Unk4	Unk4	Unk5	Unk5	Unk5	Unk6	Unk6	Unk6	Std1	Std1	Std1
C	Unk7	Unk7	Unk7	Unk8	Unk8	Unk8	Unk9	Unk9	Unk9	Std2	Std2	Std2
D	Unk10	Unk10	Unk10	Unk11	Unk11	Unk11	Unk12	Unk12	Unk12	Std3	Std3	Std3
E	Unk13	Unk13	Unk13	Unk14	Unk14	Unk14	Unk15	Unk15	Unk15	Std4	Std4	Std4
F	Unk16	Unk16	Unk16	Unk17	Unk17	Unk17	Unk18	Unk18	Unk18	Std5	Std5	Std5
G	Unk19	Unk19	Unk19	Unk20	Unk20	Unk20	Unk21	Unk21	Unk21	Std6	Std6	Std6
H	Unk22	Unk22	Unk22	Unk23	Unk23	Unk23	Unk24	Unk24	Unk24	Std7	Std7	Std7

- iii. On the results output (bottom right), check that the NTC triplicates read "N/A", indicating that these wells DID NOT AMPLIFY, on both the "9" (TEL) and the "12" (SCG) steps (switch between these using the drop menu on the middle far right of screen). Check this for both P1 and P2 files. If this is not the case, you may alter the threshold (click and drag line) on the amplification graph (top right) to place the threshold in such a place as the NTC does not reach it. HOWEVER – be careful that any resulting change in the

MMQ-PCR (Page 11 of 13)

efficiencies does not break the QC rules (see points 3-4 in previous section). If you are unable to achieve this, and your negative has a positive reading, *the plate must be repeated*.

- iv. If all your NTC pass QC...YAY... continue to the next step.
- b. Extract the Standard readings from the plate:
 - i. Begin with P1. Once you have completed steps b-d for P1, conduct these same steps for P2.
 - ii. First, add the plate number to the appropriate field at the top of the Standard excel sheet (i.e. for P1 -> "Standard P1").
 - iii. Go to the appropriate BioRad file.
 - iv. Ensure that step 9 (TEL) is selected. Highlight the "Cq" values in the results output for Std-01 through Std-07 (Do not highlight the NTC). Right click and select "copy"
 - v. Go to your excel file, and on the appropriate sheet (i.e. for P1 -> "Standard P1"), paste data into yellow fields for TEL.
 - vi. Return to the BioRad P1 file. Ensure that step 9 (TEL) is selected, take the "slope" reading (shown below Standard Curve figure) and type this value into the blue field for TEL on the same excel page.
 - vii. Return to the BioRad P1 file. Select step 12 (SCG). The relevant fields should already be highlighted. Right click and select "copy"
 - viii. Go to your excel file and, on the same sheet, paste data into yellow fields for SCG.
 - ix. Return to the BioRad P1 file. Ensure that step 12 (SCG) is selected, take the "slope" reading (shown below Standard Curve figure) and type this value into the blue field for SCG on your excel page.
 - x. The excel file has now generated intercepts for TEL and SCG (orange fields), and CV values for the T/S ratios for each dilution of the standard (green fields). *The green fields MUST be <0.1*. If you are successful, go to step d, otherwise, use step c to try to correct your errors.
- c. To eliminate points from the Standard:
 - i. If a triplicate is above this QC cutoff, you can eliminate it (either the whole triplicate or just the specific well that caused the failure) from the BioRad file calculations by entering the "plate set up" feature, highlighting the triplicate that failed, and selecting "exclude wells in analysis".
 - ii. Each time you exclude new wells, you need to repeat the previous step (i.e. step b) for BOTH TEL and SCG standards as the values will have all changed slightly. Be careful that you paste the new ct values into the correct cells and leave the appropriate cells (which correspond to the eliminated wells) blank. Hint: clear all the Ct wells and start again each time to avoid confusion when you paste the revised Cts.
 - iii. You will need to adjust the excel formulas on this page accordingly (for BOTH TEL and SCG).
 1. If you eliminated a whole triplicate: correct the intercept formula (orange cells) to exclude that point from the standard. (NB: don't forget to do the same for BOTH TEL and SCG).
 2. If you eliminated a single well: correct the appropriate formula in the ct averages (purple cells) to exclude that well. (NB: don't forget to do the same for BOTH TEL and SCG).
 - iv. You may continue to eliminate 'suspect' triplicates or individual wells to try and meet the CV QC criteria. You CANNOT have a standard that includes less than 3 full triplicate points. Also, be careful because you need your standard points to encompass the highest and lowest unknown sample reading, otherwise you are making calls outside of the range of the standard – this is not cool. If you need to eliminate more than 3 points of your standard, you should question the reliability of your standard and you should repeat this plate.
 - v. Only once you have generated a standard that meets the CV QC criteria, you may move on to extracting your sample data for that plate.
- d. Extract your sample data:
 - i. Before you begin to extract your sample data, go to your excel file and label your samples in the red field ("SUBJECT" column) on sheet "Samples P1". This will automatically fill the sample names on the subsequent pages and make it much easier for you to identify the location of a single individual on each plate (this becomes important for later QC).
 - ii. Return to the BioRad file. Go to the "Quantitation" tab (first tab, top left).

MMQ-PCR (Page 12 of 13)



- iii. On the well set up image (bottom left), unselect the standards and NTC (click and drag to highlight them in red, once you finish the selection, they will turn grey). Ensure that your unknown samples are active – if they are grey, highlight them again to make them blue (active). Now, only your 9x8 wells of unknown samples should remain blue (active).

P1 well set up image for **sample** analysis:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std1	Std1	Std1	Unk1	Unk1	Unk1	Unk2	Unk2	Unk2	Unk3	Unk3	Unk3
B	Std2	Std2	Std2	Unk4	Unk4	Unk4	Unk5	Unk5	Unk5	Unk6	Unk6	Unk6
C	Std3	Std3	Std3	Unk7	Unk7	Unk7	Unk8	Unk8	Unk8	Unk9	Unk9	Unk9
D	Std4	Std4	Std4	Unk10	Unk10	Unk10	Unk11	Unk11	Unk11	Unk12	Unk12	Unk12
E	Std5	Std5	Std5	Unk13	Unk13	Unk13	Unk14	Unk14	Unk14	Unk15	Unk15	Unk15
F	Std6	Std6	Std6	Unk16	Unk16	Unk16	Unk17	Unk17	Unk17	Unk18	Unk18	Unk18
G	Std7	Std7	Std7	Unk19	Unk19	Unk19	Unk20	Unk20	Unk20	Unk21	Unk21	Unk21
H	NTC	NTC	NTC	Unk22	Unk22	Unk22	Unk23	Unk23	Unk23	Unk24	Unk24	Unk24

P2 well set up image for **sample** analysis:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk1	Unk1	Unk1	Unk2	Unk2	Unk2	Unk3	Unk3	Unk3	NTC	NTC	NTC
B	Unk4	Unk4	Unk4	Unk5	Unk5	Unk5	Unk6	Unk6	Unk6	Std1	Std1	Std1
C	Unk7	Unk7	Unk7	Unk8	Unk8	Unk8	Unk9	Unk9	Unk9	Std2	Std2	Std2
D	Unk10	Unk10	Unk10	Unk11	Unk11	Unk11	Unk12	Unk12	Unk12	Std3	Std3	Std3
E	Unk13	Unk13	Unk13	Unk14	Unk14	Unk14	Unk15	Unk15	Unk15	Std4	Std4	Std4
F	Unk16	Unk16	Unk16	Unk17	Unk17	Unk17	Unk18	Unk18	Unk18	Std5	Std5	Std5
G	Unk19	Unk19	Unk19	Unk20	Unk20	Unk20	Unk21	Unk21	Unk21	Std6	Std6	Std6
H	Unk22	Unk22	Unk22	Unk23	Unk23	Unk23	Unk24	Unk24	Unk24	Std7	Std7	Std7

- iv. Ensure that step 9 (TEL) is selected. Highlight the “Cq” values AND “SQ” values in the results output for Unkn-01 through Unkn-24. Right click and select “copy”.
 - v. Go to your excel file, and on the appropriate sheet (i.e. for P1 -> “Samples P1”), paste data into yellow fields for TEL.
 - vi. Return to the BioRad P1 file. Select step 12 (SCG). The relevant fields should already be highlighted. Right click and select “copy”
 - vii. Go to your excel file and, on the same sheet, paste data into blue fields for SCG.
 - viii. T/S Ratio, Average, Std Dev and CV fields should be automatically calculated by the excel template formulas.
 - ix. Check the generated CV values for every triplicate. These must be <0.1 to pass QC. If any of these values fails QC, you can attempt to fix this by eliminating a single erroneous reading from the triplicate. If you have reason to believe that one of the triplicate readings is false, alter the formula in the Average, Std Dev and CV fields for ONLY that triplicate to exclude that specific cell from analysis. Check the CV value again, is it below 0.1? If not, you need to flag this sample to be repeated (I recommend going to the sheet “P1 v P2” and changing the text color for that sample to red so you are reminded not to use that data). Be aware that it is not always clear that a specific triplicate reading is the “odd one out”... if all three are different, you will not be able to solve the QC issue by eliminating a single reading, and trying to do so would not do justice to your data. That sample needs to be flagged to be repeated (as described above).
 - x. Check the intaplate variation formula at the bottom of the page. This should be <0.05.
 - xi. Once you are satisfied with the CV values of your triplicates for P1, return to step b and perform the same process for P2, completing sheets “Standard P2” and “Samples P2”.
 - xii. NOTE: Be aware that if you remove a single erroneous triplicate reading for the same sample on P1 AND P2, that results in you drawing your data from only 4 readings. This is not recommended. It is better to flag such a sample to be repeated.
 - xiii. Once you are satisfied with the CV values of the triplicates for both P1 and P2, you may assess the interplate correlation on the final sheet (“P1 v P2”).
- e. Assess interplate CVs:
- i. Go to sheet “P1 v P2” of your excel file.
 - ii. Check that fields have been automatically filled by the template formula.
 - iii. Check the interplate CVs. These must be <0.6 to pass QC. If a sample does not pass this QC step, flag it to be repeated (i.e. change the text color for that sample to red so you are reminded not to use that data).

MMQ-PCR (Page 13 of 13)

- iv. Check the overall plate variation at the bottom of the page. For the “without red” formula you will need to change the formula to only include the samples that passed all the CV checks. This should be <0.05 .
- v. For samples that were not flagged to be repeated, the orange cells indicate the final T/S ratio call for each subject. Transfer this information to the appropriate main telomere data file.