

SOP015 V1.0

1. Purpose

This document replaces SOP013 and SOP014. This document describes how to use the Tecan Freedom Evo to set up 1 primer plate with 96 primer pairs against 4 master mixes containing SYBR Green I MasterMix, and samples on a 384-well plate and run on Roche LightCycler 480.

2. Introduction

This experiment is designed to set up single 6 μ L qPCR reactions for 4 samples with 96 primers on a single primer plate. The protocol includes ordering of stock primer plate, master and working 96-well array primer plate set up, automated set up of 384 well reaction plate, Light Cycler run, and data analysis. PCR reactions are plated using Tecan Freedom Evo, and plates are run on Roche LightCycler480.

On a separate day prior to this procedure, samples should be extracted for DNA via SOP 009-SOP of DNA Isolation Using Roche MagNA Pure Compact (positive controls) or cDNA via SOP 017-SOP of QPCR mRNA Preparation (samples). Prior to running samples, primer plate should be validated by running a serial dilution of a positive control, as well as a negative control.

3. Allowable Exceptions

Rather than using 4 different samples, replicate samples may be analyzed by setting up more than one master mix per sample.

Bench copies of this protocol are allowed to be stored in two separate areas: MEJ 837A Clean room and MEJ 837 Tecan Lab bench. Bench copies must remain in designated areas at all times.

4. Responsibilities**4.1. Clean Room (MEJ 837A)**

- 4.1.1. Clean room can only be entered by PCR and sample clean persons. Therefore, those that have handled post-PCR plates/reactions, plasmids, or performed SOP 009 or SOP 016, must have showered and changed to new clothes before entering this room.
- 4.1.2. Personnel must be trained by lab manager prior to entering this room. Proper PPE must be worn at all times when in this room, this includes: booties, gloves, and lab coat.
- 4.1.3. **Weekly**, fresh 70% ethanol should be made. Date made and date expires should be marked on the bottle.
- 4.1.4. **Daily use**, the PCR cabinet, pipettes, and in use lab bench should be decontaminated with eliminase, then distilled water, and finally 70% ethanol at the end of work. PCR cabinet door should be closed and UV timer started.

4.2. Tecan

- 4.2.1. **Weekly** checklists must be completed once every week prior to first use, ensuring that the Tecan Freedom Evo is functioning properly. This must be documented in the Tecan log sheet provided.
- 4.2.2. **Daily use** checklist must also be completed for days in which the Tecan Freedom Evo is used. This must be documented in the Tecan log sheet provided. In addition, Cooled Carrier temperature should be taken and recorded according to GCLP temperature log.
- 4.2.3. **Annually**, the Tecan Freedom Evo must undergo yearly preventative maintenance to ensure no underlying issues. This must be documented and kept together with GCLP maintenance records.

4.3. Tecan Lab Space**4.3.1. Weekly:**

- 4.3.1.1. 70% ethanol should be made. Date made and date expires should be marked on the bottle.

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- 4.3.1.2. The lab bench, pipettes, minicentrifuge, and vortexes must be decontaminated with eliminase, then water, and finally 70% ethanol.
- 4.3.1.3. System liquid should be filled with water when level gets below ¼ full and should be replaced with a new jug. Once filled, the liquid container should be labeled with the date, and must sit for at least 12 hours to allow for it to settle and de-gas prior to use (for that reason, always have a backup container filled).
- 4.3.1.4. Robot plastic surface should be cleaned with water ONLY to prevent cracking.

4.3.2. Daily use:

- 4.3.2.1. Bleach for washing robot tips during run should be replaced every day in reservoir with 2% bleach made every 2 days; expiration must be marked on bottle.
- 4.3.2.2. After daily and weekly flushes, tips should be inspected visually for drips and for alignment to avoid crashing. As according to weekly check-list, syringes should be hand-tightened (bottom 2 silver syringe gearwheels and 2 white gearwheels all should tighten to the right/clockwise).
- 4.3.2.3. The system liquid should be checked as needed- heavy use may require filling of system liquid and emptying of waste more frequently.
- 4.3.2.4. System waste container should be checked for fullness periodically (every few plates it may need emptying), and disposed of by pouring down the sink and replacing with an empty backup container.
- 4.3.2.5. The system waste container should be emptied at the end of each day of use, this will prevent biofilm contamination. However, it will also be checked on designated weekly day or on the first use of the week. Any items on the checklist should always be checked off and signed with initials upon completion.
- 4.3.2.6. Robot plastic surface should be cleaned with water ONLY to prevent cracking.

4.4. Materials verified for this assay

- 4.4.1. LightCycler® 480 SYBR Green I Master (Roche Applied Science, Catalog # 04887352001)
- 4.4.2. SYBR LightCycler® 480 Multiwell Plate 384, white (Roche Applied Science, Catalog # 04729749001)
- 4.4.3. LightCycler® 480 Sealing Foil (Roche Applied Science, Catalog # 04729757001)
- 4.4.4. BioExcell® AlumaSeal II, Non-sterile (WorldWide Medical Products, Inc. Catalog # 41061019 or equivalent)
- 4.4.5. Thermo Scientific Matrix Screenmates Round-Bottom Storage Tubes, 1.4 mL, Sterile (Matrix Technologies Corp. Catalog #4252)
- 4.4.6. Sepra Seal Capmat, sterile (Matrix Technologies Corp. Catalog #4464)
- 4.4.7. Sepra Seal Pre-slit capmat, sterile (Matrix Technologies Corp, Catalog #4466)
- 4.4.8. Aerosol resistant tips for 2.5 µL, 20 µL, 200 µL, and 1000/1250 µL pipettes (GeneMate. Catalog #P-1237-10XL, P-1237-20, P-1237-200, and P-1237-1250, or equivalent)
- 4.4.9. 2.5 µL, 20 µL, 200 µL, and 1000/1250 µL micropipettes
- 4.4.10. Multichannel micropipettes capable of 20 µL-300 µL
- 4.4.11. Tris-EDTA 1x, Molecular grade, pH 8.0 (Fisher Catalog #BP2473-100 or equivalent)
- 4.4.12. 96-Channel Reservoir, Deep-Well (Matrix Technologies Corp. Catalog #1064-05-6 or equivalent)
- 4.4.13. Sarstedt® 2 mL Screw cap micro tube with cap (Sarstedt. Catalog # 72.694.007)
- 4.4.14. Microcentrifuge Tube Screw Cap with o-ring (Fisher Catalog#22-304999 or equivalent)
- 4.4.15. 0.2 mL PCR tubes (Phenix, Catalog #MPC-200F or equivalent)
- 4.4.16. Clorox Bleach (Fisher Catalog #CLO 02490 or equivalent)
- 4.4.17. Bench top minicentrifuge (Bioexpress, Catalog #C-1301-PC or equivalent)

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- 4.4.18. Eppendorf 5804R, A-2-DWP rotor: PCR plate capacity of 2200 rcf (Eppendorf, Catalog #022623508 or equivalent)
- 4.4.19. Plate shaker capable of shaking 1 minute at 750 rpm (IKA[®] MS 3 Digital Shaker. Catalog #3319000)
- 4.4.20. Scientific Industries Vortex-Genie 2, variable speed 120V (Scientific Industries, Catalog # SI-0236)
- 4.4.21. MEJ 837A Clean Room Lab coat-Valumax purple lab coat (Fisher, Catalog #23900514)
- 4.4.22. MEJ 837 Tecan side Lab coat-Valumax teal lab coat (Fisher, Catalog #23900511)
- 4.4.23. Ethanol 190 proof (Fisher, Catalog#04-355-266EA)
- 4.4.24. BC1 cell pellet positive control, 1×10^5 cells/tube (Dittmer Lab LOT#02172012 or equivalent lot)
- 4.4.25. Namalwa cell suspension positive control, 1×10^6 cells/tube (Dittmer lab Lot#08092013 or equivalent lot)
- 4.4.26. Custom Freedom Evo and accessories (Tecan Group Ltd., Switzerland)
- 4.4.27. Freedom EVOware Standard Version 2.2 software
- 4.4.28. LightCycler[®] 480 II/384 (Roche Applied Sciences, Indianapolis, IN)
- 4.4.29. Lightcycler 480 software release 1.5.0 SP3

5. Procedure

5.1. Order Primer Stock plate array

- 5.1.1. Arrays are ordered from MWG in deep well plate format, 10nmol, and salt-free or HPSF. Normalization is required as Concentration and Volume, 100 μ M in 100 μ L volume, and in TE (10mM pH 8.0) [(promo code "plate8") (100pmol/microL stock plate)]. Individual Stock primer plates should be ordered for each primer in each direction (1 deep 96-well plate each for: Primer Plate forward and Primer Plate reverse).
 - 5.1.1.1. If applicable (two array plate) Order Primer Plate 2F, and Primer Plate 2R.
- 5.1.2. For easy identification, name plates specific to the array and use 1F, 1R, 2F and 2R to distinguish between plates. Upon arrival, store primer stock plates at -80°C .
- 5.1.3. Place a hard copy of the MWG Data Sheet in the xxxxxx.
- 5.1.4. Save an electronic copy of the MWG Data Sheet on xxxxx under the specific xxxxxxxx. Label file with name of array and MWG order number.
- 5.1.5. If this is a new array, create a new excel Tecan template. Template is located in xxxxxx "Creating384 well Tecan Template_PP1_S1, 2, 3, 4". Use the "Copy 96 primers here" column to input your primer names and it will auto populate "Primer" column in accordance to the Tecan pipetting algorithm. Finally, copy that "Primer" column and paste (values) into "CreatingArray template2_TecanTemplate_PP1_S1,2,3,4" template "Primer" column. Label file "xxxxArray_MWGxxxxxxx_TecanTemplate_PPx_S1,2,3,4" and save on xxxxx under specific array folder.

5.2. Set up Master plate and Working plate:

- 5.2.1. Master Plate and Working plate should be set-up within the Clean room, following Section 4.1 responsibilities.
- 5.2.2. **Master Plate**
 - 5.2.2.1. Primer Stock plates should be thawed completely (at 4°C), mixed for 1min at 750rpm using MS3 Digital vortex plate shaker, and centrifuged at 2000 rcf for 1 minute. One Master plate will be made, containing 96 primer pairs (forward and reverse primers).
 - 5.2.2.2. Label Master matrix tube plate with: Assay name, "MWG#"* , "Master Plate[P#]", date made, initials, and "Uses"-leave space for tally marks. **Write the original Stock plate*

MWG order number for tracking and easy re-order purposes. The number usually starts with a 7xxxxx.

5.2.2.3. **Master Plate 1 (PP1 or P1)**

Reagent	Original Concentration	Volume (µL)	Final Concentration
Tris-EDTA (pH8.0) 10 mM Tris/1 mM EDTA	1x	900	0.9x
Forward Stock	100 µM	50	5 µM
Reverse Stock	100 µM	50	5 µM

5.2.2.3.1. In matrix tubes, add 900 µL sterile 1x TE to all 96 matrix tubes.

5.2.2.3.2. Add 50 µL Forward Primer stock plate and 50 µL Reverse Primer stock plate to corresponding row in matrix tubes using multichannel pipette and fresh tip box and use pipette tips to help keep your place. The concentration will be 5000nM or 5 µM (1:20 dilution). **Always check that Master plate is made using matching Forward and Reverse primer plates.*

5.2.2.3.2.1. Multiple Primer Plate Examples:

5.2.2.3.2.1.1. KSHV Dream Array (xxxx) requires 2 Master plates. PP1 will have 1F and 1R, PP2 will have 2F and 2R. *Ensure Dream Array Primer Plate 1 and Primer Plate 2 stock plates are not combined.*

5.2.2.3.2.1.2. HCMV array 2010 (xxxx) requires 2 Master plates but is only 144 primer pairs. PP1 will have “CMV plate #1” Stock plate combined with “CMV plate #2” Stock plate. PP2 will have rows A-D of “CMV plate #3” Stock plate (forward) combined with E-H of “CMV plate #3” Stock plate (reverse). A second replicate of “CMV plate #3” primer pairs will need to be repeated for Master Plate rows E-H.

5.2.2.4. If continuing to Section 5.2.3, seal all matrix tubes with foil (catalog #41061019), mix 1 minute at 750rpm using MS3 Digital vortex plate shaker, then centrifuge for 1 minute at 2000 rcf.

5.2.2.5. If applicable, repeat from Section 5.2.2.1-5.2.2.4 for Master plate 2 using Stock plate 2F and 2R.

5.2.2.6. Once finished with Section 5.2.3, seal with sterile capmat (catalog #4464).

5.2.2.7. The Master plates should be stored at -80°C and freeze/thawed max 20 times. Indicate freeze/thaw cycle by marking on matrix rack and entry onto Masterplate log sheet (outside of -80°C freezer).

5.2.3. **Working Plate**

5.2.3.1. Label Matrix tube rack with the following: Assay name, MWG#, Working Plate [P#], Date made, Date expires, initials, and “store at 4°C”.

5.2.3.2. **Working Plate (PP1 or P1)**

Reagent	Original Concentration	Volume (µL)	Final Concentration
Molecular grade water	n/a	180	n/a
Master stock (F+R) primer	5 µM	20	0.5 µM primer 0.45 mM Tris/0.045 mM EDTA

5.2.3.2.1. Add 180 µL sterile molecular grade water into each matrix tube using multichannel pipette.

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- 5.2.3.2.2. From mixed and spun down Master plate 1, mix by pipetting up and down, then remove 20 μL into new 96-well Working plate using multichannel and new box of tips. (The concentration of the Working plate will be .5 μM or 500nM (final concentration is a 1:200 dilution of stock).
- 5.2.3.2.2.1. Note: Volumes in sections 5.2.3.2-5.2.3.2.2 can be increased to accommodate specific project needs. Each array uses 3 μL of primer per Master Mix, so one can calculate volume needed for each project **plus 35% extra** for unexpected runs and Tecan overage. Minimum total volume should be 200 μL . For example a project that will require 88 Master Mixes: $(3 \mu\text{L} \times 88) \times 1.35 = 356.40 \mu\text{L}$ of each primer, so 320.76 μL sterile molecular grade water + 35.64 μL from Master Plate.
- 5.2.3.3. Seal with pre-slit capmats, mix on MS3 Digital vortex plate shaker for 1 minute at 750rpm, and centrifuge for 1 min at 2000 rcf.
- 5.2.3.4. If applicable, repeat for Working Plate 2 from Master plate 2 following steps 5.2.3.1-5.2.3.3
- 5.2.3.5. Working Plates should be stored at 4°C for a maximum on 4 weeks, mark expiration date on rack.
- 5.2.3.6. A new Working plate should be used once expired, or after a run in which the QC failed (*note- the entire plate does not need to be remade if single primers fail validation- the primers that fail can be excluded from further analysis (see analysis Section 5.9.1.9).

5.3. Quality Control (QC)

5.3.1. Negative Control (No-template control)

- 5.3.1.1. No-template control (NTC) should be run for each new Working Plate.
- 5.3.1.2. Make Master Mix as described below but replace cDNA with equivalent volume of molecular grade water.
- 5.3.1.3. A full 384-well plate, four NTC Master Mixes, should be run for each new Working Plate and/or the start of a new customer project. In excel, label as NTC rep1, NTC rep2, NTC rep3, NTC rep4.
- 5.3.1.4. NTC can be run on sample plates also by replacing sample Master Mix with NTC mix.

5.3.2. Positive Control

- 5.3.2.1. Positive control DNA/cDNA should be made from positive control cell lines, or purchased from a commercial company after sufficient validation.
- 5.3.2.2. In house cell line controls should be extracted using SOP 009 SOP of DNA Isolation Using Roche MagNA Pure Compact or SOP 017 SOP of QPCR mRNA Preparation.
- 5.3.2.3. A full 384-well plate, four positive control dilution Master Mixes, should be run for each new Working Plate and/or the start of a new customer project.
- 5.3.2.4. For the majority of arrays extracted positive controls should be diluted with molecular water and run accordingly: Neat/undiluted, 1:3, 1:9, and 1:27.

5.4. Set up Master mix and samples

- 5.4.1. Master mix contains SYBR Green I Master (2x) and cDNA. Four Master Mixes should be made for every 96 well plates (4 samples per plate). Volumes below contain enough volume (plus excess for pipetting) for 96 primer pairs-3 μL Master Mix and 3 μL primer mix per reaction. Mixes should be made in 2 mL screw top tubes as follows:

Reagent	Volume per 96 primer plate (μL)
SYBR Green I Master (2x)	374.4
cDNA or sample	20

5.4.2. Mix tubes by briefly vortexing, then spin briefly using bench top minicentrifuge for approximately 10 seconds.

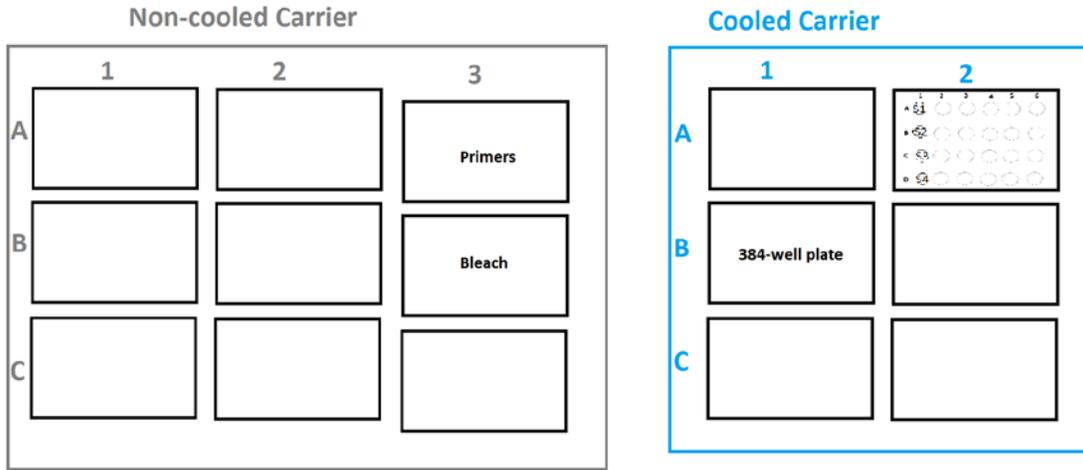
5.5. Set up PCR plate using Tecan Freedom Evo

5.5.1. Initialize and start the robot:

- 5.5.1.1. Turn on the LightCycler using the switch on the front of the machine.
- 5.5.1.2. Turn on the Tecan Freedom Evo by pressing the green button.
- 5.5.1.3. Turn on the LightCycler computer (right computer) by pressing the button on the tower.
- 5.5.1.4. Log in (keyboard for LightCycler computer is on top of desk).
- 5.5.1.5. Turn on Tecan computer (left) by button on tower.
- 5.5.1.6. Log in: (keyboard for Tecan computer is in keyboard drawer).
- 5.5.1.7. On LC computer, select LightCycler480 LIMSServer. It will load and say No Active Instrument, once Tecan is initialized it should recognize the LC.
- 5.5.1.8. Once LC software has loaded: on Tecan computer, select Evoware Standard program and log in using personal account information.
- 5.5.1.9. On Evoware, select Edit an Existing Script, then click Start Your Selection and choose the Program: MKP_96array_4samples_6ul_SOP and click the Green arrow.
- 5.5.1.10. Ensure Tecan door is closed and select "Initialize." An error message will appear indicating that the software is for testing purposes only. The window will appear behind the evoware program. Click the tab at the bottom of the screen, then select Continue. If this is not done, LightCycler will not initialize.
- 5.5.1.11. Robot will initialize and move arms, and attempt to connect to LC software. LC software should begin to load on its computer. Sometimes this does not work on the first try, and it gives an error of a failed connection- if this occurs, select Retry. If Retry does not work after 2 attempts, shut everything off in the reverse order of turning on (see steps 5.7.1.1.1-5.7.1.1.5), and restart from step 5.5.1.1.
- 5.5.1.12. Once initialized, system liquid needs to be flushed through instrument to prevent inaccurate pipetting due to air bubbles.
 - 5.5.1.12.1. Select Commands tab on left, then Flush (under Direct Commands).
 - 5.5.1.12.2. Change the Volume in Waste to 30 mLs, and select flush.
 - 5.5.1.12.3. At the first use of week and daily, always flush with at least 30 mL-three times (90 mL total).
 - 5.5.1.12.4. Always check tips following wash- if bubbles or drips are present, repeat the flush until they are gone.

5.6. Set up deck:

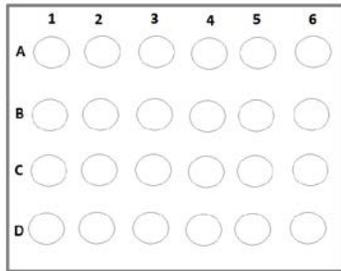
5.6.1. Deck Layout



5.6.2. MKP_96array_4samples_6uL_SOP

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- 5.6.2.1. Label a new Roche 384 well PCR plate with your initials, the date, P1 or P2 and a short identifying title. Record the barcode plate ID # in lab notebook for future reference. Place 384-well plate on black metal cooling block (stored in fridge) on Tecan Cooled Carrier, position B1 (set to 15°C).
- 5.6.2.2. Remove screw caps and place prepared Master Mix in 16-well silver metal cold block (stored in fridge) on Cooled Carrier, position A2. Samples should be placed going down row 1 (A1, B1, C1, and D1) in order of ascending concentration (if concentration is known, least concentrated samples should be position A1, and most concentrated in D1).



- 5.6.2.3. Remove pre-slit capped Working Plate Primers from 4°C fridge and mix for 1min at 750rpm using MS3 Digital vortex plate shaker, and centrifuge at 2000 rcf for 1 minute. Place desired mixed and spun-down 96 Working Plate Primers on Non-cooled Carrier, position A3 (has metal 96 well holed shelf.)
- 5.6.2.4. Place reservoir containing 75 mL of 2% bleach solution on Non-cooled Carrier, position B3.

5.6.3. Run Tecan to set up 384 well PCR plate:

- 5.6.3.1. Ensure that deck is correct, then select Run.
- 5.6.3.2. When run screen opens, select Run.
- 5.6.3.3. Robot will begin with a wash, then aliquot 3 µL master mix of SYBR and samples across the plate, followed by 3 µL primers. Robot will bleach and flush between samples and between each primer.
- 5.6.3.4. If liquid error is detected, robot computer will give options and unlock the door. First, select Retry Detection, as sometimes this is a result of a detection error that can be resolved by trying again. If retry does not work 3 times, select Go To Z Max. If Z Max does not work, be sure to take note of the well that gave the error for analysis, there probably is no liquid left in that well.
- 5.6.3.5. When robot is complete, select Close on the software. The 384 well plate should be removed from the black cold block and sealed CAREFULLY with a clear sealing film from Roche. If no other samples need to be run, primer plates should returned to the 4°C fridge. Leftover master mixes can be discarded, and cold blocks should be returned to the fridge.
- 5.6.3.6. Once sealed the 384 well plate should be mixed for 1min at 750rpm using MS3 Digital vortex plate shaker, and centrifuged at 2000 rcf for 1 minute.

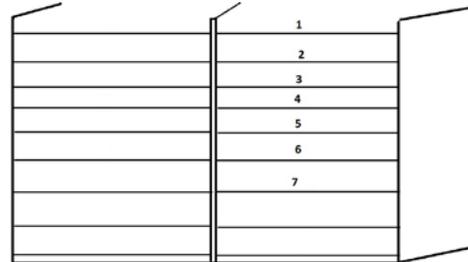
5.7. Run 384 well PCR plate on Roche LightCycler 480**5.7.1. Automated (robot-fed) PCR run**

- 5.7.1.1. After the Tecan has set-up the 384 well PCR plate, the robot will need to be restarted in order to reestablish the connection. Shut down the robot in the following order:
 - 5.7.1.1.1. Shut down Tecan Evoware software. Move arms to home position.

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- 5.7.1.1.2. Shut down LightCycler software. An error may come up indicating that user should ensure that Evoware is shut down prior to shutting down the LightCycler. Ensure evoware is shut down, then continue to shut down LightCycler.
- 5.7.1.1.3. Turn off Tecan computer, then LightCycler computer.
- 5.7.1.1.4. Turn off Tecan (hold button in until light goes off).
- 5.7.1.1.5. Turn off LightCycler.
- 5.7.1.2. Wait for a minute to rest, then turn everything on again following steps 5.5.1.1-5.5.1.8.

- 5.7.1.3. In the meanwhile, place mixed and spun down 384 well PCR plate in the top right position in the hotel with additional plates under it if there are more than one to run, maximum of 7 plates. The plate will fit nicely into the groove of the hotel, and the notch should face out.



- 5.7.1.4. On Evoware, choose Edit an Existing Script, then select the program: `mkp_HOTEL_SOPArray6ulSYBR_Protocol`.
Note: Samples submitted to the Core for testing must use only the SOP approved programs.
- 5.7.1.5. Verify by checking Tecan protocol that the LightCycler program is correct: "Macro SOP Array 6uL SYBR Protocol-Admin"
- 5.7.1.6. Complete set-up from Section 5.5.1.10-5.5.1.12.4.
- 5.7.1.7. Select Run. On run screen that opens, select run again.
- 5.7.1.8. An alert will pop up ensuring that you verified the LightCycler program. If you have, click OK.
- 5.7.1.9. A "set variable" command will pop up asking the number of plates. Enter the number of plates in hotel and select Ok. The robot will load the PCR plate into the LightCycler. The PCR machine will initialize and begin running in a few minutes.

5.7.1.10. Cycling Conditions

Cycle Name		Cycles		Analysis Mode	
Pre-amplification		1		none	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	
95	None	00:05:00	4.8		
Cycle Name		Cycles		Analysis Mode	
Amplification		40		Quantification	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	
95	None	00:00:15	4.8		
62	Single	00:01:00	2.5		
Cycle Name		Cycles		Analysis Mode	
Melting Curve		1		Melting Curves	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	
95	None	00:00:05	4.8		
60	None	00:01:00	2.5		
95	Continuous	-	0.11	5	
Cycle Name		Cycles		Analysis Mode	
Cool		1		none	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	
50	None	00:05:00	2.5		

5.7.1.11. After plates are run, they will be returned to the same position in the hotel. (Run 384 well PCR plates will be slightly warped.)

5.7.1.12. Post-run 384 well PCR plates should be stored in -80°C freezer until no further testing is required, then they can be discarded as biohazardous waste.

5.8. LightCycler 480 data calculation and analysis

5.8.1. Export data:

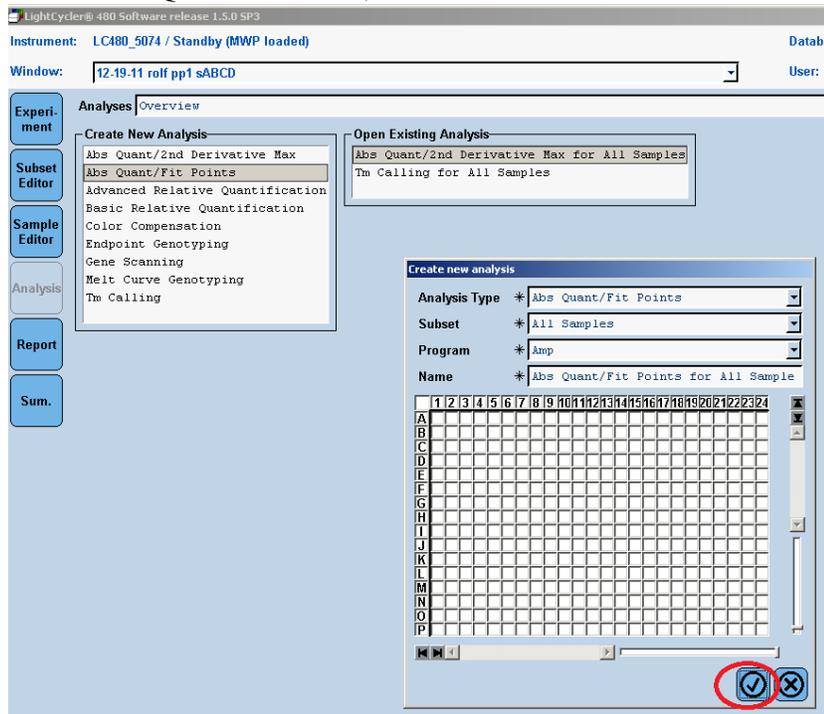
5.8.1.1. Open the LightCycler 480 SW 1.5 software (after shutting down Evoware and Tecan computer and closing the LightCycler 480 LIMSServer software if run integrated with Tecan) and log in.

5.8.1.2. To locate the file: on top where Overview is displayed, select dropdown arrow and choose Navigator. Either find the file manually or use search Query tool. Note: Plates run through integration with Tecan will be named DDMMYYYY.0 for the first plate and the decimal will increase by 2 for each subsequent plate. Double click desired file to open.

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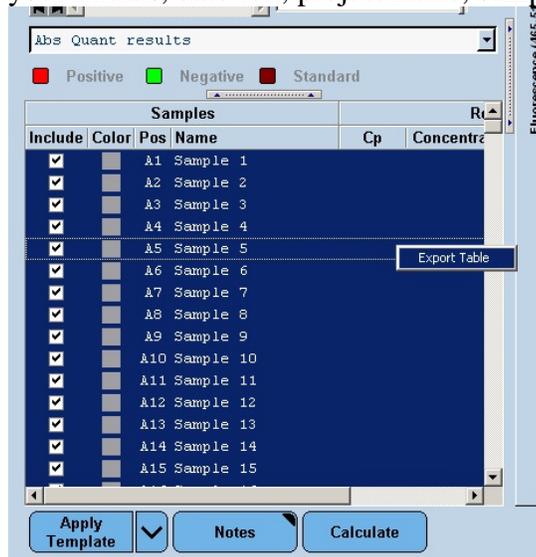
5.8.1.3. Verify that the run cycling conditions, file name and date are correct, then select the analysis tab.

5.8.1.4. Select Abs Quant/Fit Points, then click the check mark.



5.8.1.5. Click calculate and the software will automatically set the threshold for noise background subtraction. As needed, manually adjust the noise band above noise threshold and adjust cycle range to just above background noise level, then select calculate again. Save any additional analysis under a different name within the software by “re-naming” the analysis file in case one needs to come back to it.

5.8.1.6. To export data, right click on bottom right analysis and select export table. Table will export and save as a .txt file, which can be later opened with Excel. Save file name with your initials, date run, project name, samples name, and P1 or P2.

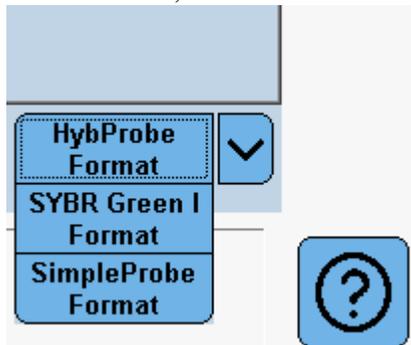


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- 5.8.1.7. Next select Show Overview (image looks like a Family Tree) and select Tm Calling to get melting temperature data. Click check mark.



- 5.8.1.8. On bottom right, change format to SYBR Green I. Then click calculate. Export table in same manner as 5.6.1.6. For convenience, save it as the exact same name as Abs Quant/Fit Points data, with additional indication that it is the Tm or melt curve within the file name.



5.9. Analysis:

- 5.9.1.1. Data should be analyzed using the spreadsheet designed to interpret data (see additional excel appendix). Always open as Read Only to avoid changes being made to original spreadsheet.
- 5.9.1.2. Highlight the columns to the left of the grey dividing column. Custom sort (with headers) the template by row then column, check that the wells are in ascending order A1 to A24 all the way to P24.
- 5.9.1.3. Open both Tm and Ct exported files with excel. Highlight Cp (crossing point) data on Abs Quant/Fit Values spreadsheet and paste into Ct/Cp Column on array specific spreadsheet, ensuring that the wells are sorted in the same order on both sheets. Use Find/Replace function to replace all blank cells with 40.
- 5.9.1.4. Repeat for Tm1 and Tm2 data using Tm/melt file. Use Find/Replace function to replace all blank cells with 0.
- 5.9.1.5. Type in Sample names in the fillable column to the right (A1, B2, C3, D4), recorded in lab notebook, and excel will integrate data into appropriate location. (No spaces or characters other than underscore.)
- 5.9.1.6. Complete other fillable columns: Date, Plate name (ie. NTC, STD, samples), and Plate ID#.
- 5.9.1.7. Custom sort by Primer, then sample.
- 5.9.1.8. If applicable:
- 5.9.1.8.1. Calculate median for replicates of primers.
 - 5.9.1.8.2. Sample Concentration. Log10 for all samples greater than zero. Formula is =log10(Sample Concentration).
 - 5.9.1.8.3. Calculate all R2 using the formula =RSQ(log10Sample Concentration, Cp).
 - 5.9.1.8.4. Calculate all CV using the formula =STDEVA(Cp)/MEDIAN(Cp).
- 5.9.1.9. **Determining quality of run (QC):**
- 5.9.1.9.1. Any primer for which water (NTC) comes up prior to cycle 40, data should be excluded due to potential contamination.
 - 5.9.1.9.2. Positive controls should come up prior to cycle 40 and have a Tm for all Neat/undiluted controls.

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- 5.9.1.10. File should be saved under a new name and located in xxxx for PI analysis/approval.
(Original Template file should be a read only and not modified.)

6. References

- 6.1. LightCycler 480 Basic Software Version 1.2. Roche Diagnostics GmbH, 2006 June.
- 6.2. MagNA Pure Compact Operator's Manual Instrument Version 1.0 Software Version 1.1. Roche Diagnostics GmbH, Mannheim Germany, version 1.3.
- 6.3. Vandesompele et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*. 2002, 3(7).

7. Appendix

7.1. Quick Guide: Primer Stocks (Clean room)

Stock Plate Arrives 100 μ M in 100 μ L

Master Plate (Matrix tubes, sterile capmat)

Reagent	Original Concentration	Volume (μ L)	Final Concentration
Tris-EDTA	1x	900	0.9x
Forward Stock	100 μ M	50	5 μ M
Reverse Stock	100 μ M	50	5 μ M

Label: Assay Name, MWG stock order #, “Master Plate” plate#, Date made, initials, “uses- “

Working Plate (Matrix tubes, pre-slit capmat)

Reagent	Original Concentration	Volume (μ L)	Final Concentration
Molecular grade water		180	
Master stock (F+R)	5 μ M	20	0.5 μ M

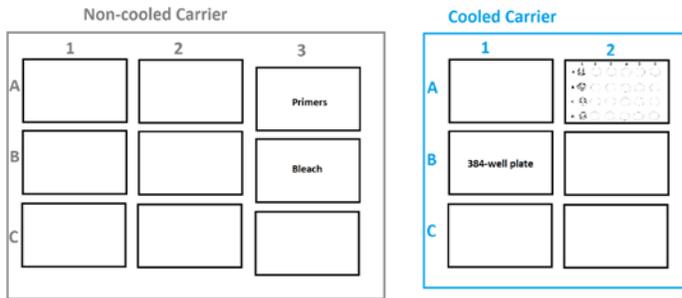
Label: Assay Name, MWG stock order #, “Working plate” plate#, Date made, Date expires (4 weeks), initials, “store at 4°C”

7.2. Master Mix and Tecan Set-up Quick Guide

Master Mix

Reagent	Volume per 96 primer plate (µL)
SYBR Green I Master (2x)	374.4
cDNA or sample	20

384-well Tecan Program: MKP_96array_4samples_6ul_SOP



Automated PCR Run Tecan Program: mkp_HOTEL_SOPArray6ulSYBR_Protocol

Macro: Macro SOP Array 6uL SYBR Protocol-Admin

Cycle Name		Cycles		Analysis Mode	
Pre-amplification		1		none	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	
95	None	00:05:00	4.8		
Cycle Name		Cycles		Analysis Mode	
Amplification		40		Quantification	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	
95	None	00:00:15	4.8		
62	Single	00:01:00	2.5		
Cycle Name		Cycles		Analysis Mode	
Melting Curve		1		Melting Curves	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	
95	None	00:00:05	4.8		
60	None	00:01:00	2.5		
95	Continuous	-	0.11	5	
Cycle Name		Cycles		Analysis Mode	
Cool		1		none	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	
50	None	00:05:00	2.5		

7.3. Tecan Weekly and Daily Checklist

		Tecan Checklist																																		
		Month: _____																																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31				
Weekly:																																				
	Empty Liquid Waste																																			
	Fill System Liquid																																			
	Flush 3x <30ml																																			
	Tighten Syringes																																			
	Wipe bench with 70% ethanol/10% bleach																																			
	Initials																																			
	Daily:																																			
	Make 2% bleach																																			
	Check tips																																			
	Flush <30ml																																			
	Initials																																			

7.4. Array's Excel Analysis Spreadsheet Templates
(see excel appendix file)