

SOP #: SOP015

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Approval: **Approved By**

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Staff Signatures: (signing below indicates that you have read this SOP and understand the material contained in it)

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Table of Contents

1. Purpose.....	3
2. Introduction.....	3
3. Allowable Exceptions	3
4. Responsibilities	3
4.1. Clean Room	3
4.2. Tecan.....	3
4.2.4. Annually.....	4
4.3. Tecan Lab Space	4
5. Materials verified for this assay	5
6. Procedure	7
6.1. Order Primer Stock plate array	7
6.2. Set up Master plate and Working plate:.....	7
6.3. Quality Control (QC)	8
6.4. Set up Master mix and samples.....	9
6.5. Set up PCR plate using Tecan Freedom Evo	9
6.6. Run 384-well PCR plate on Roche LightCycler 480	13
6.7. Data Acquisition from LightCyler 480	15
6.7.1. Export data:	15
6.7.2. Importing at desktop	15
6.8. Import Data into Excel and Analysis:	17
6.8.1. Excel Data.....	17
6.8.2. Determining quality of run (QC):	17
6.8.3. Compiling all Project Data into One Excel.....	18
7. References.....	18
Appendix.....	19
Quick Guide: Primer Stocks (Clean room)	19
Master Mix and Tecan Set-up Quick Guide	20
Tecan weekly and daily checklist	21
Tecan Freedom Evo Labware teaching tracking.....	22

1. Purpose

This document describes how to use the Tecan Freedom Evo to set up 1 qPCR plate from 1 primer plate of 96 primer pairs against 4 master mixes containing SYBR Select Master Mix, 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, LightCycler 480 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 positive control plate (a serial dilution of a positive control), and a negative control plate (a series of clean room water samples).

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 must remain in designated areas at all times.

4. Responsibilities

4.1. Clean Room

- 4.1.1. Clean room can only be entered by PCR and sample clean persons. Therefore, those that have handled samples, post-PCR plates/reactions, plasmids, or performed anything in other lab spaces, 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 clean room specific lab coat.
- 4.1.3. **As needed**, fresh 70% ethanol should be made. Date made and date expires should be marked on the bottle (7 day expiration).
- 4.1.4. **Daily use:** After 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. **Review Appendix Checklist**
- 4.2.2. **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.

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- 4.2.3. **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.
- 4.2.4. **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.
- 4.2.4.1. **The Tecan must be re-taught before any use to avoid crashing. If there is trained staff in house, Vironomics Core can perform this. If not, before the Tecan technician leaves they should perform the following with observation by a core staff member.**
 - 4.2.4.1.1. See Appendix for the Labware teaching tracking to record changes
 - 4.2.4.1.2. First the 384-well teaching plate should be put on the black cooled block and plated on the carrier. Push the block to the upper left before teaching.
 - 4.2.4.1.3. Then manually adjust the tiny positioning screws for each tip to center within the 384-wells. This is time consuming but must be done.
 - 4.2.4.1.4. Then check the appropriate labware included in our two most used scripts.
 - 4.2.4.1.4.1.ms_VL_SOP8V5_5primers_24samples_384_18p9ul_09112017
 - 4.2.4.1.4.2.MKP_96array_4samples_6ul_SOP_09112017

4.3. Tecan Lab Space

- 4.3.1. 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: gloves and Tecan lab space specific lab coat.
- 4.3.2. **Weekly:**
 - 4.3.2.1. 70% ethanol should be made. Date made and date expires should be marked on the bottle (7 day expiration).
 - 4.3.2.2. The lab bench, pipettes, minicentrifuge, and vortexes must be decontaminated with eliminase, then water, and finally 70% ethanol.
 - 4.3.2.3. 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). Flush the system immediately after switching system liquid on the Tecan.
 - 4.3.2.3.1.1. First EVOware screen and click “edit an Existing Script”, choose any.
 - 4.3.2.3.1.2. Select Commands tab on left, then Flush (under Direct Commands).
 - 4.3.2.3.1.3. Change the Volume in Waste to 30 mLs, and select flush.
 - 4.3.2.3.1.4. Flush with at least 30 mL-three times (90 mL total).
 - 4.3.2.4. On an as-needed-basis, acrylic safety panels should be cleaned with water ONLY to prevent cracking.
 - 4.3.2.5. 2% Contrad followed by DI-water can be used on labware carriers. Triple check that the carriers have not moved from their original position when performing cleaning.
- 4.3.3. **Daily use:**
 - 4.3.3.1. Bleach for washing Tecan tips during run should be replaced every day in reservoir with 2% bleach made every 2 days; expiration must be marked on bottle.
 - 4.3.3.2. After daily and weekly Maintenance Washes, tips should be inspected visually for drips and for alignment to avoid crashing.
 - 4.3.3.3. **As according to weekly check-list, syringes should be hand-tightened (bottom 2 silver syringe thumbscrews and 2 white thumbscrews all should tighten to the right/clockwise).**

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- 4.3.3.4. The system liquid should be checked as needed- heavy use may require filling of system liquid and emptying of waste more frequently.
- 4.3.3.5. 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.
- 4.3.3.6. The system waste container should be emptied at the end of each day of use, this will prevent biofilm contamination.
- 4.3.3.7. Any items on the checklist should always be checked off and signed with initials upon completion.

4.3.4. End of Project:

- 4.3.4.1. Clean carriers and worktable with 2% Contrad followed by DI-water.
- 4.3.4.2. CAUTION: Make sure that the carriers are in the correct positions after cleaning and have not shifted. The non-cool carrier has marks on the worktable to indicate if correct now.
- 4.3.4.2.1. Note: Do not clean in/on the wash stations.

5. Materials verified for this assay

- 5.1. Nitrile Gloves, Kimberly-Clark Spring Green Powder Free (Fisher, Catalog #43438 or equivalent)
- 5.2. Wypall (Fisher, Catalog #1979001 or equivalent)
- 5.3. Eliminase, small (Fisher, Catalog #4-355-31)
- 5.4. Blue absorbent pad/diaper/underpads with waterproof moisture barrier (VWR, Catalog # 56616-018 or equivalent)
- 5.5. Microcentrifuge tube racks (Fisher, Catalog #05-541-1 or equivalent)
- 5.6. SYBR Select Master Mix (Life Technologies, Catalog # 447290)
- 5.7. LightCycler® 480 Multiwell Plate 384, white (Roche Applied Science, Catalog # 04729749001)
- 5.8. LightCycler® 480 Sealing Foil (Roche Applied Science, Catalog # 04729757001)
- 5.9. BioExcell® Foil Film for Cold Storage (WorldWide Medical Products, Inc. Catalog # 41061019 or equivalent)
- 5.10. Thermo Scientific Matrix Screenmates Round-Bottom Storage Tubes, 1.4 mL, Sterile (Matrix Technologies Corp. Catalog # 4252)
- 5.11. Septra Seal Capmat, sterile (Matrix Technologies Corp. Catalog # 4464)
- 5.12. Septra Seal Pre-slit capmat, sterile (Matrix Technologies Corp, Catalog # 4466)
- 5.13. 2.5 µL, 20 µL, 200 µL, and 1000 µL pipettes (Eppendorf, Catalog # 3120000909 or equivalent)
- 5.14. Aerosol resistant tips for 2.5 µL, 20 µL, 200 µL, and 1000 µL pipettes (Genesee, Catalog #24-401, 24-404, 24-412, 24-430, or equivalent)
- 5.15. 2.5 µL, 20 µL, 200 µL, and 1000/1250 µL micropipettes (Eppendorf, Catalog # 3120000909 or equivalent)
- 5.16. Multichannel micropipettes capable of 20 µL-300 µL
- 5.17. Molecular Biology Grade Water (Cellgro, Catalog # 46-000-CI or equivalent)
- 5.18. Tris-EDTA 1x, Molecular grade, pH 8.0 (Fisher Catalog # BP2473-100 or equivalent)
- 5.19. 96-Channel Reservoir, Deep-Well (Matrix Technologies Corp. Catalog # 1064-05-6 or equivalent)
- 5.20. 2.0 ml flat bottom microcentrifuge tubes, autoclaved, max speed 18,000 x g (Fisher, Catalog #02-681-343 or equivalent)
- 5.21. 1.5 mL “conical” microcentrifuge tubes, autoclaved, max speed 30,000 x g (Fisher, Catalog #02-681-339 or equivalent)
- 5.22. Microcentrifuge Tube with o-ring screw cap, autoclaved (Fisher, Catalog #2-681-360 or equivalent)
- 5.23. 0.2 mL PCR tubes (Phenix, Catalog # MPC-200F or equivalent)
- 5.24. Clorox Bleach (Fisher Catalog # CLO 02490 or equivalent)

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- 5.25. Contrad 70 labware cleaner (Decon Labs, Catalog #1002)
- 5.26. Bench top minicentrifuge (Bioexpress, Catalog # C-1301-PC or equivalent)
- 5.27. Hermle Labnet Z306 Centrifuge without rotor (Phenix, Catalog # C-0306)
- 5.28. Hermle Labnet swing out rotor for microplates (Phenix, Catalog # C-0036-26)
- 5.29. Plate shaker capable of shaking 1 minute at 750 RPM (IKA® MS 3 Digital Shaker. Catalog # 3319000)
- 5.30. Scientific Industries Vortex-Genie 2, variable speed 120V (Scientific Industries, Catalog # SI-0236)
- 5.31. Clean Room Lab coat-Valumax Extra-Safe, knit collar purple lab coat (Fisher, Catalog #23900514)
- 5.32. Tecan side Lab coat-Valumax Extra-Safe, knit collar teal lab coat (Fisher, Catalog # 23900511)
- 5.33. Ethanol 190 proof (Fisher, Catalog # 04-355-266EA)
- 5.34. BC1 cell pellet positive control, 1×10^6 cells/tube
- 5.35. Namalwa cell suspension positive control, 1×10^6 cells/tube
- 5.36. Custom Freedom Evo and accessories (Tecan Group Ltd., Switzerland)
- 5.37. Freedom EVOware Standard Version 2.7 software
- 5.38. LightCycler® 480 II/384 (Roche Applied Sciences, Indianapolis, IN)
- 5.39. LightCycler 480 software release 1.5.1 SP3
- 5.40. “Fly 2.0 10x” *Drosophila melanogaster* Amplicon in pIDTBlue (Integrated DNA Technologies)

6. Procedure

6.1. Order Primer Stock plate array

- 6.1.1. Arrays are ordered from MWG, now called Eurofins Genomics, in shallow well plate format, 10 nmol, and salt-free. Normalization is required as Concentration and Volume, 100 μ M in 100 μ L volume, and in TE (10 mM pH 8.0) (100 pmol/microL stock plate)]. Make sure to select Dry Ice shipping for the order (if available).
- 6.1.1.1. 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.)
 - 6.1.1.1.1. If applicable (two array plate) Order Primer Plate 2F, and Primer Plate 2R.
 - 6.1.1.2. For easy identification, name plates specific to the array and use 1F, 1R, 2F and 2R to distinguish between plates.
- 6.1.2. Upon arrival, store primer stock plates in clean room -20°C. Log on the freezer tracking log.
- 6.1.3. Place a hard copy of the MWG Data Sheet in the GCLP binder with received date and initials.

6.2. Set up Master plate and Working plate:

- 6.2.1. Master Plate and Working plate should be set-up within the Clean room, following Section 4.1 responsibilities.
- 6.2.2. **Master Plate**
 - 6.2.2.1. Primer Stock plates should be thawed completely (at 4°C), mixed for 1 min 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).
 - 6.2.2.2. 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.*
 - 6.2.2.3. **Master Plate 1 (PP1 or P1)**

Reagent	Original Concentration	Volume (μ L)	Final Concentration
Tris-EDTA (pH 8.0) 10 mM Tris/1 mM EDTA	1x	900	9 mM Tris/0.9 mM EDTA
Forward Stock	100 μ M	50	5 μ M
Reverse Stock	100 μ M	50	5 μ M

- 6.2.2.3.1. In matrix tubes, add 900 μ L sterile 1x TE to all 96 matrix tubes.
- 6.2.2.3.2. Add 50 μ L Forward Primer stock plate and 50 μ L 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 5000 nM or 5 μ M (1:20 dilution). **Always check that Master plate is made using matching Forward and Reverse primer plates.*
- 6.2.2.3.2.1. Multiple Primer Plate Examples:
 - 6.2.2.3.2.1.1. KSHV Dream Array 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.*
 - 6.2.2.3.2.1.2. HCMV array 2010 requires 2 Master plates but is only 144 primer pairs. PP1 will have “CMV plate #1” Stock plate combined with “CMV plate #2”

File: SOP S015-V2.0 WEB ONLY SOP of Tecan 96 Primer (1 plate) Array with 4 Master Mixes

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.

6.2.2.4.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. .

6.2.2.5.If applicable, repeat for Master plate 2 using Stock plate 2F and 2R.

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

6.2.2.7.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 -20°C freezer).

6.2.3. Working Plate

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

6.2.3.2.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 0.9 mM Tris/0.09 mM EDTA

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

6.2.3.2.2. 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 0.5 µM or 500 nM (final concentration is a 1:200 dilution of stock).

6.2.3.2.2.1. 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 µL x 88) x 1.35= 356.40 µL of each primer, so 320.76 µL sterile molecular grade water + 35.64 µL from Master Plate.

6.2.3.3.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. .

6.2.3.4.If applicable, repeat for Working Plate 2 from Master plate 2.

6.2.3.5.Working Plates should be stored at 4°C for a maximum on 4 weeks, mark expiration date on rack.

6.2.3.6.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).

6.3. Quality Control (QC)

6.3.1. Negative Control (No-template control)

6.3.1.1.No-template control (NTC) should be run for each new Working Plate.

File: SOP S015-V2.0 WEB ONLY SOP of Tecan 96 Primer (1 plate) Array with 4 Master Mixes

6.3.1.2. Make Master Mix as described below but replace cDNA with equivalent volume of molecular grade water.

6.3.1.3. 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.

6.3.1.4. NTC can be run on sample plates also by replacing sample Master Mix with NTC mix.

6.3.2. Positive Control

6.3.2.1. Positive control DNA/cDNA should be made from positive control cell lines, or purchased from a commercial company after sufficient validation.

6.3.2.2. 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.

6.3.2.3. 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.

6.3.2.4. 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.

6.3.2.4.1. *Dilution series: 12µL of sample + 24µL molecular water*

6.4. Set up Master mix and samples

6.4.1. Master mix contains SYBR Select Master Mix (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 Select Master (2x)	374.4
cDNA or sample	20

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

Note: 2015 successfully ran cDNA as low as 3ng/uL (customer reported)

6.5. Set up PCR plate using Tecan Freedom Evo**6.5.1. Initialize and start the Tecan:**

6.5.1.1. Turn on the Tecan Freedom Evo by pressing the green button.

6.5.1.2. Turn on Tecan computer

6.5.1.3. On Tecan computer, select Evoware Standard program and log in using personal account information.

6.5.1.4. On Evoware, select “Run maintenance”. Click “Start your selection”, then click “Run”.

6.5.1.5. Follow user prompts and ensure each step of daily maintenance has been completed. The Tecan will then initialize and move arms, then flush 50 mL.

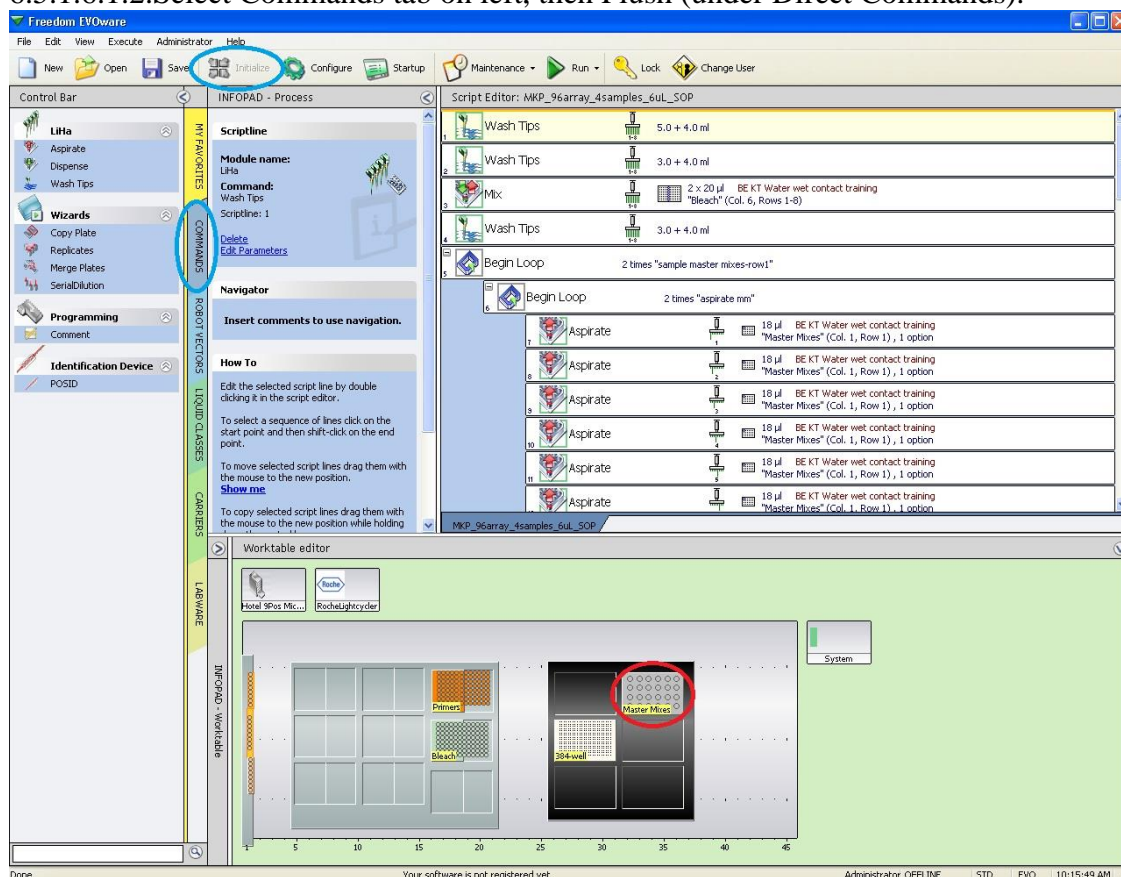
6.5.1.6. Note user prompt to watch for bubbles in syringes. Click okay. As Tecan pipettes full volume up and down, watch clear section of syringes to see if any bubbles are visible. If bubbles, there is air in the pipetting lines which must be flushed out. Repeat maintenance wash after this script completes.

6.5.1.6.1. Note: Record in Tecan maintenance manual if this does not clear up. If 3x maintenance wash does not work, then may need to do a real flush.

6.5.1.6.1.1. Return to first screen and click “edit an Existing Script”, choose any.

File: SOP S015-V2.0 WEB ONLY SOP of Tecan 96 Primer (1 plate) Array with 4 Master Mixes

6.5.1.6.1.2. Select Commands tab on left, then Flush (under Direct Commands).



6.5.1.6.1.3. Change the Volume in Waste to 30 mLs, and select flush.

6.5.1.6.1.4. Flush with at least 30 mL-three times (90 mL total).

6.5.1.6.1.5. Consider having Tecan come service that channel if issue continues.

6.5.1.7. Note user prompt to check pipette tips for droplets after the final tip wash. If droplets are visible, repeat 1_Daily_Wash_110717.

6.5.1.8. Complete the Tecan Weekly and Daily Checklist.

6.5.2. Set up deck:

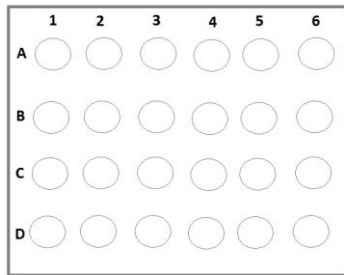
6.5.2.1. Select circle next to Run Existing Script

6.5.2.2. Select **script**

6.5.2.3. 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 (see Appendix for easy lab notebook tracking). Place 384-well plate on black metal cooling block (stored in fridge) on Tecan Cooled Carrier, position B1 (set to 15°C).

6.5.2.4. 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).

File: SOP S015-V2.0 WEB ONLY SOP of Tecan 96 Primer (1 plate) Array with 4 Master Mixes



6.5.2.5. Remove pre-slit capped Working Plate Primers from 4°C fridge and mix for 1 min at 750rpm using MS3 Digital vortex plate shaker, and centrifuge at 2000 rcf for 1 minute. Remove plastic lid and place desired mixed and spun-down 96 Working Plate Primers on Non-cooled Carrier, position A3 (has metal 96 well holed shelf.)

6.5.2.6. Place reservoir containing 75 mL of 2% bleach solution on Non-cooled Carrier, position B3.

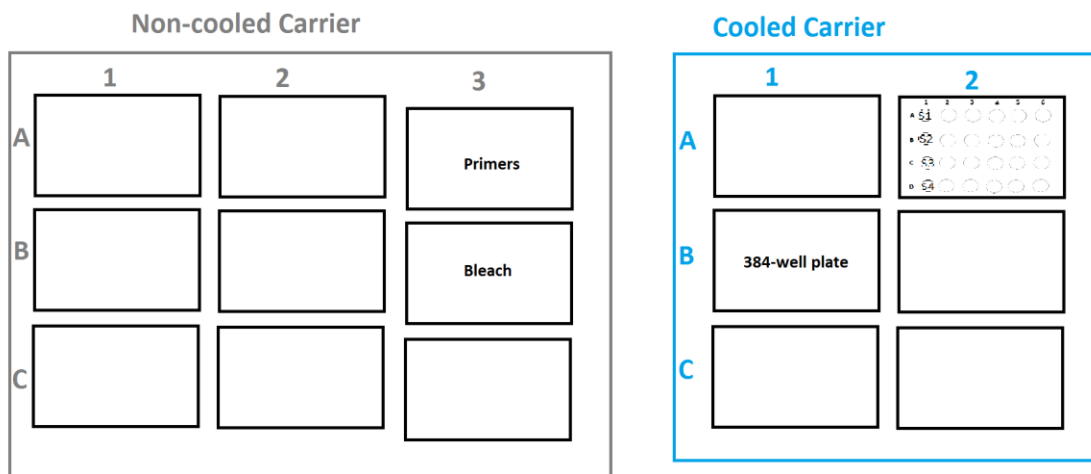
Note: filling to the line inside the reservoir is a good estimate.

6.5.3. Run Tecan to set up 384-well PCR plate:

6.5.3.1. Ensure that deck is correct.

6.5.3.2. Gently push all blocks, reservoirs, etc to their upper left position on the carriers. This is especially important with the small wells of the 384-well plate.

6.5.3.3. Deck Layout



6.5.3.4. When run screen opens, select Run. **The run will take ~1 hour 15 minutes** (depending on volume of primer plate).

6.5.3.5. Tecan will begin with a wash, then aliquot 3 μ L master mix of SYBR and samples across the plate, followed by 3 μ L primers. Tecan will bleach and flush between samples and between each primer.

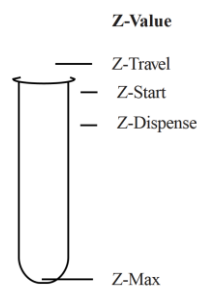
6.5.4. **If liquid error** is detected, Tecan computer will give options and unlock the door. First, select *Retry Detection*, as sometimes this is a result of a detection error that can be resolved by trying again. If retry does not work 3 times, record in notebook the tip and sample/primer the error is occurring with then

6.5.4.1. Select Retract to Z travel and visually check if there is enough liquid left (~50 μ L of liquid)

6.5.4.1.1. (a) if not, record and exclude data as there wasn't adequate volume at the beginning- select Go to Z Max, record in notebook and in analysis

File: SOP S015-V2.0 WEB ONLY SOP of Tecan 96 Primer (1 plate) Array with 4 Master Mixes

- 6.5.4.1.2. (b) if there is, remove from Tecan, recap, and minicentrifuge, then Retry Detection. If this still produces an error, select Go to Z max, record in notebook and in analysis-check the tube at the very end to see if empty.
- 6.5.4.2.Note: It has been noticed that sometimes when told to go to Z-max, it actually pipets air, this is why we record carefully and may to exclude the data depending on expertise.
- 6.5.4.2.1. Example of what “Z” directions are (as taught to Tecan by advanced user)
- 6.5.4.2.1.1.**Z-Travel:** Height that the tips can safely move so as not to hit the tube
- 6.5.4.2.1.2.**Z-Start:** Height it can begin trying to detect liquid
- 6.5.4.2.1.3.**Z-Dispense:** Height it is programed to aliquot liquid at (changes with tube type)
- 6.5.4.2.1.4.**Z-Max:** Location of the bottom of the tube, generally taught slightly above the bottom so the tips do not get broken

**6.5.4.3.Post Tecan Completion**

- 6.5.4.3.1. When script 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.
- 6.5.4.3.2. If no other samples need to be run, primer plates should be returned to the 4°C fridge. Leftover master mixes should be kept in the 4°C fridge until the experiment has been completed (and then can be discarded in biohazard waste), and cold blocks should be returned to the fridge.
- 6.5.4.3.3. At the end of the day (or as necessary during the day) the waste should be emptied by bringing the new waste bottle under the worktable next to the filled bottle, removing the lid of the new bottle and placing it upside down on the computer table, spinning the **BOTTOM** of the filled waste bottle to remove the lid without kinking the tubing, moving the tubes into the empty waste bottle, spinning the **BOTTOM** of the empty waste bottle to tighten the lid, and putting the lid on the full waste bottle. Move the full waste bottle to the sink and empty.
- 6.5.4.3.4. Make sure the extra carboy of system liquid (Dracor deionized water) is full, as it will need to de-gas overnight before use. To fill, carefully walk the carboy as close to the sink as possible, remove the lid from the large carboy and place it upside down on the side of the sink, remove tubing from below the sink (carefully, don't touch this to anything unnecessary or put it down anywhere except the purple box under the sink), connect the tubing to the Dracor deionized water output and place the opposite side in the carboy, and turn on the deionized water. **IMPORTANT, STAY NEXT TO THE CARBOY WHILE FILLING. Do not flood the lab.** Fill the carboy up to the point where the carboy begins to narrow. Remove the tubing, and carefully wind it up to replace under the sink. Put the lid on the large carboy and allow it to de-gas overnight.

File: SOP S015-V2.0 WEB ONLY SOP of Tecan 96 Primer (1 plate) Array with 4 Master Mixes

6.6. Run 384-well PCR plate on Roche LightCycler 480

6.6.1. Turn on the LightCycler using the switch on the machine.

6.6.2. Turn on the LightCycler computer.

6.6.3. Open LightCycler 480 SW version of software. Wait until software shows it has finished its start-up process.

6.6.4. Click “new experiment from macro”

6.6.5. Select appropriate macro per below

6.6.5.1. Cycling Conditions “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		

6.6.5.2. Cycling Conditions “Macro UDG 96 Array 6uL SYBR Protocol_2018_03”, validated for KSHV-DAv2018 Primary Plate

Cycle Name		Cycles		Analysis Mode	
UDG Activation		1		none	

File: SOP S015-V2.0 WEB ONLY SOP of Tecan 96 Primer (1 plate) Array with 4 Master Mixes

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
50	None	00:02:00	4.8	
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	

6.6.6. If verification of the LightCycler program needs to be done, between plates one will need to go to the Navigator and look under System Admin for the program. The conditions should be as below (listed along right hand part of the screen).

6.6.7. Note: Samples submitted to the Core for testing must use only the SOP approved programs.

6.6.8. LC480 will most likely require the plate to be in the instrument before it continues, use the door open/close button on the side facing the wall.

6.6.9. Create an experiment file name. Begin with the date, project name, assay, primer plate, details, and your initials. This file defaults to the experiments folder under System Admin

6.6.10. Wait around to confirm the run begins and the temperature is increasing before walking away.

6.6.11. Due to the LC480 plate door opening into the Tecan, the Tecan may need to be paused in order to load and unload the LC480.

File: SOP S015-V2.0 WEB ONLY SOP of Tecan 96 Primer (1 plate) Array with 4 Master Mixes

- 6.6.12. Pause the Tecan from the Tecan computer software, generally best to do this at a DI-water wash step.
- 6.6.13. Make sure not to bump anything in the Tecan nor pass over any open tubes/plates.
- 6.6.14. Remember to un-pause (play) the Tecan after the LC480 door is closed.
- 6.6.15. Store completed plates in a ziplock, change gloves before closing ziplock, DO NOT remove the air from bag as this could cause aerosol amplicon contamination if the plate has become unsealed.
 - 6.6.15.1. After done for the day, store plate(s)-ziplock in designated dirty freezer.
- 6.6.16. After the last plate has been run, save runs onto lab USB flash drive and transfer to available computer for analysis (see section below).
- 6.6.17. Turn off the LC480 instrument.
- 6.6.18. Close the software and turn off the LC480 computer.
- 6.6.19. After the Tecan has set up-the last 384-well PCR plate of the day, the Tecan will need to be shut-down in the following order:
- 6.6.20. Shut down Tecan Evoware software. Yes, move arms to home position.
- 6.6.21. Turn off Tecan (hold button “on” until light goes off).
- 6.6.22. Turn off the Tecan computer.

6.7. Data Acquisition from LightCycler 480

6.7.1. Export data:

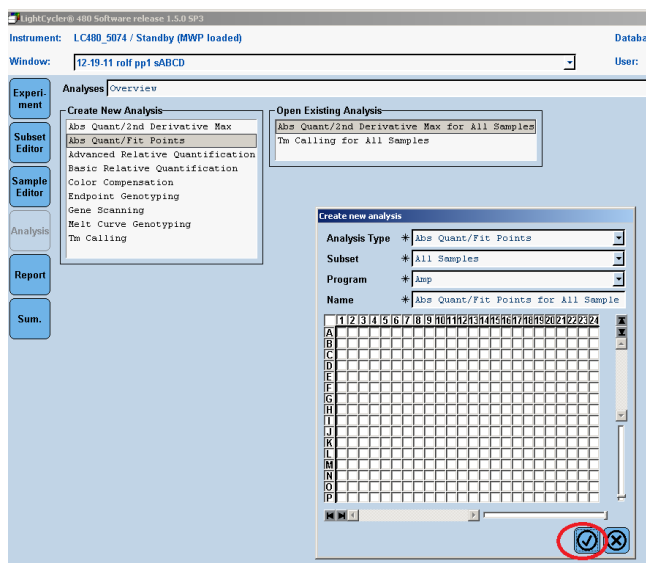
- 6.7.1.1. Open the LightCycler 480 SW 1.5.1 software (after shutting down Evoware and Tecan computer and closing the LightCycler 480 LIMSServer software if run integrated with Tecan) and log in.
- 6.7.1.2. To locate the file: click on the icon on the right with a star in a circle (Navigator).
- 6.7.1.3. Select “Batch Export”.
- 6.7.1.4. Either find the file manually or use search Query tool. Data for plates run manually will be under “Experiments”. Click to select. Use Ctrl to select non-consecutive files or Shift to select consecutive files.
- 6.7.1.5. Move files for export over to the right hand side of the sheet using “>>”. Select “Next”. Choose the destination directory for data (flash drive). Select “Next”. Leave defaults as is and select “Next”. Files will be exported one at a time, when file export is completed you will be given a list of # of files exported and # of files that failed to export. Failure to export may be because the file has previously been exported and already is in the selected destination. Select “Done”.
- 6.7.1.6. Close the program prior to ejecting the flash drive.
- 6.7.1.7. Move to separate computer for analysis.

6.7.2. Importing at desktop

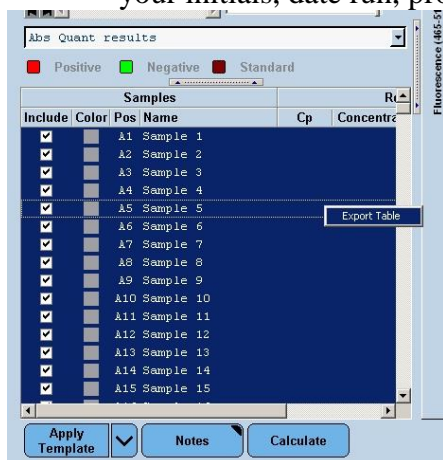
- 6.7.2.1. In lab notebook, please record which computer this is done at for future reference as there are several computers with the virtual software. Better yet, save the final analyzed .ixo into the network project folder so anyone can access.
- 6.7.2.2. If not already done, create a new folder for this project.
- 6.7.2.3. Under Navigator, click “Batch Import”
- 6.7.2.4. Click add and find the appropriate file folder on USB (Note: it will import all files from that folder, make sure to clean out the folder/USB after use). Click ok, then next.
- 6.7.2.5. Choose the specific project folder, click next.

File: SOP S015-V2.0 WEB ONLY SOP of Tecan 96 Primer (1 plate) Array with 4 Master Mixes

- 6.7.2.6. Leave default options, click next.
- 6.7.2.7. Complete prompts until it confirms that the files have been transferred.
- 6.7.2.8. Back at Navigation open the double-click first file to be analyzed, confirm the plate number first.
- 6.7.2.9. Verify that the run cycling conditions, file name and date are correct, then select the analysis tab.
- 6.7.2.10. Click Analysis along the left.

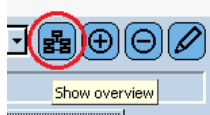


- 6.7.2.11. Select Abs Quant/Fit Points, then click the check mark.
- 6.7.2.12. Click calculate and the software will automatically set the threshold for noise background subtraction. As needed, manually adjust the noise band above noise threshold and adjust cycle range to just above background noise 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.
- 6.7.2.13. 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.

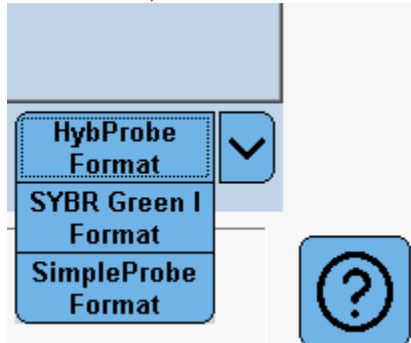


- 6.7.2.14. Next select Show Overview (image looks like a Family Tree) and select Tm Calling to get melting temperature data. Click check mark.

File: SOP S015-V2.0 WEB ONLY SOP of Tecan 96 Primer (1 plate) Array with 4 Master Mixes



- 6.7.2.15. 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.



- 6.7.2.16. Export Tm data as did before for Cp.
- 6.7.2.16.1. Note: recent software patches have had a glitch in being able to select the data. May need to click the square at the intersection of A and 1 until the full plate of data shows.

6.8. Import Data into Excel and Analysis:

6.8.1. Excel Data

- 6.8.1.1. Data should be analyzed using the spreadsheet designed to interpret specific array data (see additional excel appendix). Always open as Read Only to avoid changes being made to original spreadsheet. **Note: It is very important not to change these headings of columns as the R code depends on them.**
- 6.8.1.2. Highlight the columns to the left of the fillable columns (exclude the A1, B1, C1, D1 etc columns on the left). Custom sort (with headers) the template by Well (or row then column), check that the wells are in ascending order A1 to A24 all the way to P24. 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.
- 6.8.1.3. Repeat for Tm1 and Tm2 data using Tm/melt file. Use Find/Replace function to replace all blank cells with 0.
- 6.8.1.4. 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 or dash.)
- 6.8.1.5. Complete other fillable columns: Date, Plate name (ie. NTC, STD, samples), and Plate ID#.
- 6.8.1.6. Select the columns left of the fillable columns again, Custom sort by Primer, then Sample.

6.8.2. Determining quality of run (QC):

- 6.8.2.1. Negative control Any primer for which water (NTC) comes up prior to cycle 40, data should be excluded due to potential contamination.

File: SOP S015-V2.0 WEB ONLY SOP of Tecan 96 Primer (1 plate) Array with 4 Master Mixes

- 6.8.2.2. Positive controls should come up prior to cycle 40 and have a T_m for all Neat/undiluted controls.
- 6.8.2.3. If applicable, run an R² analysis on the positive control dilution series. At least 60% of the primers should be positive for the undiluted.
 - 6.8.2.3.1. Sort columns to the left of the fillable columns. Custom sort by Primer the Sample.
 - 6.8.2.3.2. Use a column to the right of the fillable columns to calculate concentration (undiluted=1, 1:3=1/3, 1:9=1/9, 1:27=1/27) and log₁₀Sample concentration (=log₁₀(concentration))
 - 6.8.2.3.3. formula =RSQ(log₁₀SampleConc, Cq)
 - 6.8.2.3.4. Primers with R² above 0.8 is good.
- 6.8.2.4. RTminus The RTminus samples/pools should not be positive for anything, if they are this indicates that there is gDNA contamination. Analysis can adjust for this as “background level” but it should be noted that the pool samples are generally diluted 1:10, see mRNA protocol notes.
- 6.8.2.5. Samples Samples with human cells should at least be positive for a few of the human genes. This demonstrates the extraction (DNA or cDNA) was successful even if there is no viral gene expression. Some assays have built in negative control genes, in which case these should be negative.

6.8.3. Compiling all Project Data into One Excel

- 6.8.3.1. For ease with R code it is best to compile all the data into one excel sheet. This will also assist with sorting and not accidentally moving the fillable columns.
- 6.8.3.2. File should be saved under a new name and located in networked project folder.
- 6.8.3.3. Once the full project is complete. Create a new excel sheet.
- 6.8.3.4. Copy the headers from the left of the fillable columns.
- 6.8.3.5. Copy all data to the left of the fillable columns.
- 6.8.3.6. Paste via Values and Formatting. This will keep all colors and actual values instead of formulas.
- 6.8.3.7. Repeat for each 384-well plate data that belongs to the project (NTC, STD, samples plates).
- 6.8.3.8. One of the first checks analyst will do with R Code is if there are rows in multiples of 384, so let analysis group know ahead of time if for reason a full 384-well plate data is not included.
- 6.8.3.9. Send this compiled excel summary to Dr. Dittmer for analysis/approval.

7. References

- 6.9. LightCycler 480 Basic Software Version 1.2. Roche Diagnostics GmbH, 2006 June.
- 6.10. MagNA Pure Compact Operator's Manual Instrument Version 1.0 Software Version 1.1. Roche Diagnostics GmbH, Mannheim Germany, version 1.3.
- 6.11. 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).

File: SOP S015-V2.0 WEB ONLY SOP of Tecan 96 Primer (1 plate) Array with 4 Master Mixes

Appendix

Quick Guide: Primer Stocks (Clean room)

Stock Plate Arrives 100 μ M in 100 μ L TE (pH 8.0, 10 mM Tris, 1 mM EDTA)

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

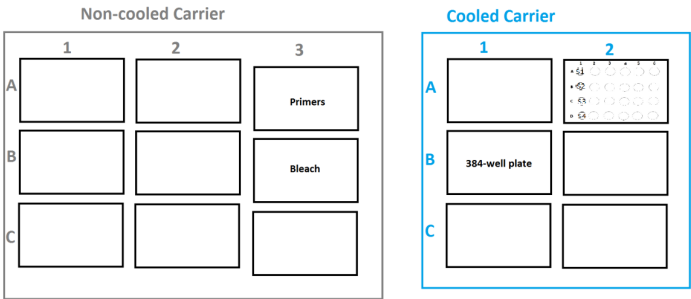
Label: Assay Name, MWG stock order #, “Working plate” plate#, Date made, Date expires (4 weeks), initials, “store at 4°C”

Master Mix and Tecan Set-up Quick Guide

Master Mix

Reagent	Volume per 96 primer plate (µL)
SYBR Select Master Mix	374.4
cDNA or sample	20

384-well Tecan Program: MKP_96array_4samples_6ul_SOP_09112017



Tecan weekly and daily checklist

File: SOP S015-V2.0 WEB ONLY SOP of Tecan 96 Primer (1 plate) Array with 4 Master Mixes
Tecan Freedom Evo Labware teaching tracking

File: Tecan Freedom Evo Labware teaching tracking

Tecan Freedom Evo Labware teaching tracking |

Effective 3/2013
2017-11-09

Date	Labware name (exact)	Program used	Carrier Position	Notes	Position Before	Position After	Initials