

Supplemental Material B.

Real-time Quantitative Polymerase Polymerase Chain Reaction

A. Operation and Programming of Roche LightCycler

1. Computer login

- a) Login computer with username: “admin” and password: “Xagorarakis59”.
- b) Double-click the “Lightcycler Software 4.05” icon. Login window will appear. Username is “lab” and password is “Xagorarakis59”.

2. PCR reaction programming

- a) Click the “new” tab and then “Lightcycler Experiment” icon. Select the “run” window at the left side of the screen.
- b) Enter “50” in “Seek Temperature” and “3 Ch.” in “instrument type”.
- c) Under the “Programs” section, use the “+” and “-” tab to add and delete the program. Edit the program section according to the following table:

Table A-4. Cycles and analysis mode of PCR program

Program Name	Cycles	Analysis Mode
Denaturation	1	None
Amplification	45-50	Quantification
Cooling	1	None

- d) Under the “Amplification Temperature Target” section, Edit the program section according to the following table:

Table A-5. Target temperature and duration of each PCR stage

Program	Target (°C)	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)	Acquisition Mode
Denaturation	95	00:15:00	20	0	0	0	None
Amplification	95	00:00:10	20	0	0	0	None
	55	00:00:30	20	0	0	0	None
	72	00:00:15	20	0	0	0	Single
Cooling	40	00:00:30	20	0	0	0	None

- e) Click the “Samples” icon on the left side of the screen.
- f) Enter a number for total samples that would be analyzed.

- g) Enter the sample name according to the sequence of the samples that would be placed in the carousel. Enter the replicated samples under the “Repl. Of” column.
- h) Enter any reminder information about the samples in “Sample Note” section.
- i) Click the “Analysis Type” icon on top of the Sample Data screen. Select “Absolute Quantification”.
- j) If the sample is the standard, select “Standard” under the “Sample Type” column. Otherwise; select it as “Unknown”.
- k) Enter the standard concentrations (copies/L or copies/reaction) under the “Concentration” column.
- l) Double check all the entries and put down any reminder information in the “Run Notes” section under the Run Window.
- m) Save the program by clicking the “Save” icon on top of the screen.

B. Quantification of virus gene by real-time PCR

1. Program the LightCycler real-time PCR system
2. Wear lab coat, and glove.
3. Disinfect the PCR Preparation Station (i.e., Misonix PCR Prep Station) using 10% Bleach, 70% EtOH, UV light and/or DNAZap (Ambion).
4. Thaw 5X LightCycler Master Mix, 10 μ M forward primer, 10 μ M reverse primer, 10 μ M probe, PCR-grade water, samples and standards.
5. Prepare a PCR super mix according to the following table (based on Roche Applied Science LightCycler TaqMan Master lab handbook).

Table A- 6. Components of the PCR mix for one reaction in the real-time PCR assay

Ingredients		Volume /reaction	Total reactions	Total volume	Final concentration
LightCycler Master Mix* (5X)		4 μ L	n+2	4 \times (n+2)	1X
Forward primer (10 μ M stock)		0.5 μ L	n+2	0.5 \times (n+2)	0.25 μ M
Reverse primer (10 μ M stock)		0.5 μ L	n+2	0.5 \times (n+2)	0.25 μ M
probe (10 μ M stock)		0.3 μ L	n+2	0.3 \times (n+2)	0.15 μ M
PCR-grade water		9.7 μ L	n+2	9.7 \times (n+2)	---

Total final volume	15 μ L	n+2	15 \times (n+2)
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* Ready-to-use hot start PCR reaction mix (after pipetting 10 μ L Enzyme from 1a vial into 1b Reaction Mix vial)

- a) A super mix for n+2 reactions (n = total samples) was prepared in a 1.5 mL centrifuge tube at the PCR Prep Station.
 - b) The super mix with a total volume of 15 \times (n+2) μ L (i.e., 15 μ L per reaction) contained 4 \times (n+2) μ L of 5X LightCycler Master Mix (LightCycler[®] TaqMan[®] Master, Roche Applied Science, Mannheim, Germany), 0.5 \times (n+2) μ L of 10 μ M forward primer, 0.5 \times (n+2) μ L of 10 μ M reverse primer, 0.3 \times (n+2) μ L of 10 μ M probe, and 9.7 \times (n+2) μ L of PCR-grade water.
 - c) Mix the super mix completely and centrifuge shortly to remove drops from lid or sidewall of the centrifuge tube.
6. Dispense 15 μ L of the super mix into each LightCycler capillary placed in the LightCycler Centrifuge Adapter.
 7. Add 5 μ L of DNA template (i.e., DNA extracts, standard solutions, positive control, and/or negative control) into each assigned capillary at the Sample Adding Station (the bench behind the PCR Prep Station).
 8. Seal each capillary with a plastic stopper, and transform all capillaries to the LightCycler Sample Carousel.
 9. Centrifuged the carousel (with capillaries filled with PCR reaction mix) using the LightCycler Carousel Centrifuge.
 10. Load the carousel into the Roche LightCycler 1.5 and start real-time PCR program by clicking the “Start Run” icon.
 11. Put reagents, samples, and standard solution back to -20 $^{\circ}$ C freezer. Clean-up the PCR Preparation Station and the Sample Addition Station.

C. Analyzing the data using Lightcycler Software 4.05

1. Saving the standard curve

The standard curve can be saved for future quantification. To save the standard curve, click “Standard curve (In Run)” and select “Save as external”. Name the standard curve accordingly.

In order to use previously saved standard curve for quantification of unknown samples, each batch of the reaction should contain at least a duplicated standard

with the same concentration as one point of the saved standard curve.

2. Absolute quantification

After all the PCR reaction is completed, click the “Analysis” icon and select Absolute Quantification (provided there is at least a duplicated standard in the run). Report file can be created by select “Report”. The detail of the report can be tailored by selecting the parameter at the left side of the screen.