Diagnosis of HCMV by real time PCR

Context: A 58-year-old man, which has been on peritoneal dialysis since 2015 for renal failure, was transplanted in May 2020. The patient had negative CMV serology at the time of the allograft. You will analyze this patient's blood samples from the summer of 2020.

You will work in groups of two students (and one group of three). Each group is responsible for analyzing two samples from this patient, and collectively, we will analyze the results of the 12 samples.

DNA sample preparation (Previously done)

DNA extraction was performed using a commercial kit on 1mL of patient's blood. DNA was eluted in 50 µL of elution buffer.

Q1: If you quantify 1.2x10^4 copies of the CMV genome in the 50 µL of elution, how many copies were originally present in the blood sample (in copies/mL)?

Amplification of the UL123 CMV gene by real-time PCR on a 96-Well BioRad CFX device: Detection by TaqMan® probe

This technique is adapted from the article: *Monitoring cytomegalovirus infection in adult and pediatric bone marrow transplant recipients by a real-time PCR assay performed with blood plasma. Leruez-Ville M, Ouachée M, Delarue R, Sauget AS, Blanche S, Buzyn A, Rouzioux C.* ***J Clin Microbiol****. 2003 May; 41(5):2040-6.*

Q2: Describe briefly the principle and the use of TaqMan® probes.

The accuracy and reproducibility of real-time PCR quantification results depend on the quality of pipetting at each step of the experiment. The PCR technique is especially sensitive to contamination. Therefore:

- Handle micropipettes with care (precision instruments), and use filter tips
- Change the tip to dispense each DNA matrix, premix or primer
- Wear a lab coat and gloves

Distribution of PCRs by two/three-person group

Each group will perform 8 PCRs (according to the table below):

- **4 PCRs for 4 standard curve points (performed in triplicate by the other groups in the class).**
- **4 PCRs corresponding to two patient samples (performed in duplicate by your group).**

Q3: What is the purpose of the replicates? Are they biological or technical replicates?

The total reaction volume is **15 µl**: **5 µl** of DNA template + **10 µl** of PCR premix.

Q4: If you quantify 0.3x10^5 copies of the CMV genome in the 15 µL of reaction, what was the initial concentration of your sample?

Group	Samples	U_L123 standard curve
1	Sample **1**: 02/07/2020 Sample **7**: 02/08/2020	Standards: 10^7, 10^5, 10^3 and 10^1
2	Sample **2**: 09/07/2020 Sample **8**: 04/08/2020	Standards: 10^7, 10^5, 10^3 and 10^1
3	Sample **3**: 16/07/2020 Sample **9**: 08/08/2020	Standards: 10^7, 10^5, 10^3 and 10^1
4	Sample **4**: 23/07/2020 Sample **10**: 12/08/2020	Standards: 10^6, 10^4, 10^2 and NTC
5	Sample **5**: 25/07/2020 Sample **11**: 18/08/2020	Standards: 10^6, 10^4, 10^2 and NTC
6	Sample **6**: 28/07/2020 Sample **12**: 22/08/2020	Standards: 10^6, 10^4, 10^2 and NTC

PCR premix preparation

The mix has been pre-prepared and distributed to each group.

PCRs will be performed using the "Applied Biosystems®" reagent which contains: Buffer, $MgCl_2$, dNTP (including dUTP instead of dTTP) and Taq polymerase.

Q5: What is the role of each component?

- **Ready-to-use PCR premix is supplied to you (see table below). Each group needs 10 µL x 8 = 80µL of premix; 85 µL are provided (for 8.5 PCR).**

Q6: Why is a mix prepared for 8.5 reactions whereas only 8 reactions are needed?

	Volume for 1 reaction	Volume for 8.5 reactions	Final concentration
Master mix 2X	?	?	1X
Forward primer CMV (10 µM)	?	?	300 nM
Reverse primer CMV (10 µM)	?	?	300 nM
Probe FAM-TAMRA CMV (5 µM)	?	?	200 nM
Water up to 10 µL	?	?	
TOTAL	?	?	

*The mix has been pre-prepared and distributed to each group. Note that the total reaction volume is 15µL (**5 µl** of DNA template + **10 µl** of PCR premix).*

Q7: Complete the missing values in the table for PCR premix preparation

An 8-well strip has been distributed to you. Note the presence of the letters A and H which help you to orient your strip. Fill the wells according to the attached table (page 4) and your group number.

Add 10 µL of premix per well in the 8 wells without changing the tip. Do not worry if the liquid does not reach the bottom of the tube.

CMV standards (4 wells per group)

From 10^1 to 10^7 copies per 5 µL (10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7) and negative controls (NTC: Non-Template Control).

Q8: What is the goal of the NTC sample? What results do you expect?

Add 5 µL of these standards to the mix. Each group is responsible for **four standards** of the standard curve (one replicate), but data from all groups will be used to obtain triplicate values.

You must load 5 µL of the 4 standard samples in the wells of the strip according to the attached table (page 4).

Q9: What is the goal of the standard curve? Why are we using it?

Patient samples (4 wells per group)

1. According to the table (page 4): in two wells, load 5 µL of each sample (two per group)
2. Close the wells tightly using the strip of caps, pressing hard.
3. Go to the Transcriptome Platform.
4. Centrifuge the strips at 3,000 rpm for 10 seconds to allow the liquid to go to the bottom of the tubes.
5. Load the strips in the Biorad CFX 96 device according to the attached table and start the run.

Biorad CFX 96 setup

Initial steps		Denaturation	Annealing and Elongation
2 min	10 min	5 sec	20 sec
50 °C	95 °C	95 °C	60 °C
AmpErase activation (UNG)	Hotstart DNA polymerase activation	45 cycles	

Q10: What is the role of the UNG enzyme?

Analysis of results

Q11: Determine the slope of the standard curve.

	Expected	Observed
R (efficiency)	1 (0.98-0.99)	
Slope	-3.32 (-3.30->3.6)	

Representation of results

For each of your sample:

Q12: Express the results in CMV copies number per 5 µL of DNA

Q13: Express the results in copies number per mL of blood sample

Q14: Express the results in Log_{10} per mL of blood sample

Sample layout for CMV PCR on Biorad CFX Maestro device

	Group 1		Group 2		Group 3		Group 4		Group 5		Group 6	
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 10^7		Standard 10^7		Standard 10^7		Standard 10^6		Standard 10^6		Standard 10^6	
B	Standard 10^5		Standard 10^5		Standard 10^5		Standard 104		Standard 10^4		Standard 10^4	
C	Standard 10^3		Standard 10^3		Standard 10^3		Standard 10^2		Standard 10^2		Standard 10^2	
D	Standard 10^1		Standard 10^1		Standard 10^1		**NTC**		**NTC**		**NTC**	
E	**Sample 1**		**Sample 2**		**Sample 3**		**Sample 4**		**Sample 5**		**Sample 6**	
F	**Sample 1**		**Sample 2**		**Sample 3**		**Sample 4**		**Sample 5**		**Sample 6**	
G	**Sample 7**		**Sample 8**		**Sample 9**		**Sample 10**		**Sample 11**		**Sample 12**	
H	**Sample 7**		**Sample 8**		**Sample 9**		**Sample 10**		**Sample 11**		**Sample 12**	