4-4-2023 SL

Cytomegalovirus DNA Detection and Quantification Protocol By Light Cycler-PCR with Hybridization Probes

A. Sample DNA purification

Using QIAamp DNA Blood Mini Kit manual procedure (see attached manufacturer protocol).

B. Preparation of real-time PCR master mix

Using Roche LightCycler FastStart DNA Master Hybprobe Kit and CMV primers and probes.

1. Thaw out MgCl2, TIB CMV01 and 02 primers and CMV03 and 04 probes on ice. The probes should keep from light before using. Thaw out one vial 1a (red cap) and one vial 1b (colorless cap) of the LightCycler-FastStar Enzyme. Thaw out one vial of CMV standard (2000copies/ul) as well.
2. Briefly centrifuge all the thawed reagents.
3. Pipet 60ul from vial 1b into vial 1a and mix gently by pipetting up and down. Do not vortex.
4. Relabel the vial 1a +1b enzyme mix and date it. (if for later use, store at -70 º C)
5. Mix all the regents as below (for 33 reactions):

25mM MgCl2	66ul
100uM CMV 01	4.95ul
100uM CMV 02	4.95ul
100uM CMV 03	1.98ul
100uM CMV 04	1.98ul
1a +1b Enzyme mix	66ul
PCR grade H2O	184.14ul
Total PCR master mix	330ul

6. Aliquot PCR master mix 62ul (6x reactions) each in a 1.5ml tube, store at -70º C and protect the master mix from the light.

C. Preparation of PCR-sample mixture and amplification

1. Thaw out PCR master mix as needed. For each sample, use 10ul of master mix and 10ul of sample DNA. For each run, should include positive standard and negative control. Up to total 32 reactions can be monitored in a single run.
2. Place the Light Cycler Capillaries in to the Light Cycler Centrifuge Adapters in a cooling block. Always keep cooling block at 4º C before use.
3. Add 10ul master mix and 10ul sample DNA or controls to each capillary, close the caps (stoppers).

4. Carefully transfer the capillaries into the carousel, place the carousel into a LC centrifuge and spin. Check the capillaries If any volume was less or more than about 20ul, re-prepare the mixture.
5. Turn on the Light Cycler and computer, and start the self-test as needed. (If the self-test fail, please contact the Roche technical support at 1-800-262-4911).
6. Place the carousel into the Light Cycler .
7. Start running the program file "CMV-Roche-LC-Run Protocol".

Denaturation:	95º C	for	10 min.	
Amplification:	95º C	for	10 sec.	
(45 cycles)	54º C	for	14 sec.	
	72º C	for	15 sec.	
Melting Curve:	95º C	for	10 sec.	
	40º C	for	10 sec.	
	85º C	for	0 sec.	Slope 0.2 º C/s
	45º C	for	20 sec.	

8. Enter the sample's names and the standard control's copy number (we used 20,000 copies/10ul of TIB plasmid as standard).

D. Data Analysis:

1. **Qualitative Analysis:** Click on Analysis button to open Tm Calling Screen. Select Melting Curve program to proceed to the Melting Curve Analysis Screen. If the sample has a same Melting peak as the positive control, it was identified as positive. For samples such as tissue and stool only use qualitative analysis method.

2. **Quantitative Analysis**:
   1. Click on Analysis button to proceed to Absolute Quantification Screen. Select the external standard curve, open "CMV-Roche-std" in data file. Print out this page as a record and calculate the actual CMV copy number of samples (copies/ml) using one of the following formulas:
      - PBS **NOT** added = copy no. x 50
      - Bone Marrow samples diluted **1:2** in PBS = copy no. x 100
      - PBS **added** as needed = copy no. x 10,000/ volume of original sample in µl
      - CSF = copy no. x 300 (if using Chelex extraction).
      - If urine sample using QIAamp viral RNA kit to extract = copy no. x 42.9.

   Value Ranges:
   Negative = TND
   Positive (no qnt) = <1,000 copies/ml
   Positive (qnt) = 1,000-1,000,000 copies/ml
   Positive (over qnt) = >1,000,000 copies/ml