4-3-2023 SL

Epstein-Barr Virus DNA Detection and Quantification Protocol By Light Cycler-PCR with Roche Primers and Probes Kit

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 Roche EBV LC primers and probes kit.

1. Thaw out Roche EBV one of each EBV primer Pair, EBV Probe 1, EBV Probe 2, Probe C-1 and Probe C-2 on ice. The probes should keep from light before using. Thaw out two vials 1a (red cap) and two vials 1b (colorless cap) of the LightCycler-FastStar Enzyme. Thaw out one vial of EBV standard (2000copies/ul) as well.
2. Briefly centrifuge all the thawed reagents.
3. Pipet 60ul from vial 1b into vial 1a and mix two together gently by pipetting up and down. Do not vortex.
4. Relabel the vial 1a +1b mixture and date it. (if for later use, store at -70 º C)
5. Mix all the regents as below (for 58 reactions):

H2O, PCR grade	116ul
Primer Pair	116ul
EBV probe-1	58ul
EBV probe-2	58ul
Probe C-1	58ul
Probe C-2	58ul
Ia+Ib Mixture	116ul
Total PCR master mix	580ul

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. 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 "EBV-Roche-LC-Run Protocol".

Denaturation:	95º C	for	10 min.	
Amplification:	95º C	for	10 sec.	
(45 cycles)	55º C	for	15 sec.	.
	72º C	for	15 sec.	
Melting Curve:	95º C	for	0 sec.	
	40º C	for	60 sec.	
	80º C	for	0 sec.	Slope 0.1 º C/s
	40º 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 "EBV-Roche-std" in data file. Print out this page as a record and calculate the actual EBV 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