A QUANTITATIVE REAL TIME PCR ASSAY FOR DETECTING EBV VIRUS IN MULTIPLE SAMPLE TYPES

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INTRODUCTION

We are developing a real time quantitative PCR assay to detect EBV in serum, plasma, whole blood, tissue and spinal fluid. Real time PCR, with its intrinsic quantitative capacity, is an excellent method for measuring EBV viral load. Epstein Barr virus is a member of the Herpesvirus family, with a tropism for B lymphocytes, where it establishes latency. In transplant settings, it causes post transplantation lymphoproliferative disorder (PTLD). High doses of immunosuppressive drugs allow the virus to escape the immune system, which normally keeps the latent virus in check. Symptoms of PTLD can mimic those of organ rejection, leading to increased immunosuppression, when a decrease in dosage is actually necessary.

The primers and probes for this assay are supplied by Epoch/Nanogen and target a region of the BFRF1 gene. This EBV assay design has the probe-binding site overlapping one of the primer binding sites by five nucleotides. Dilutions of a plasmid containing the cloned amplicon are used as standards. A new standard curve is generated and stored with each new lot of EBV reagents or Taq polymerase.

Extraction:
Extractions were performed on the Qiagen 9604 Robot using the recommended protocol. Internal control plasmid is added to the lysis buffer to give a final concentration in the extracted samples of 100 copies per µL (±1000 copies per 10 µL extracted sample).

Amplification and detection:
The EBV primer and probe reagents are provided by Epoch/Nanogen Biosciences and include primers and probe for amplification of an internal control plasmid. In contrast to previously described Eclipse probes, which have the fluorescent dye on the 3’ end, the EBV and internal control probes used in this assay have the fluorescent dye (FAM or PY59, respectively) on the 5’ end, with the quenching molecule on the 3’ end. This inverted orientation provides for lower background fluorescent signal and increased solubility. The EBV probe is labeled at the 5’ end with FAM and a minor groove binding molecule (MBG). The 3’ end of the EBV probe is labeled with a non-fluorescent quencher (NFQ). The internal control probe is also labeled at the 5’ end with a non-fluorescent dye, PY59, and the MBG. The primers in the probe sequences indicate guaninesites that have been chemically modified to prevent probe aggregation and quenching of the fluorescent dye. Red indicates primer sequences, green indicates EBV probe sequence, purple indicates internal control probe sequence, and nucleotides in blue indicate regions where a primer and probe sequence overlap. The “K” in the EBV target sequence indicates the position of a polymorphism (either a T or a G); the “M” in the corresponding EBV downstream primer indicates the position of a degenerate base.

The EBV primers for this assay have a non-template, 12 nucleotide AT-rich tail added to their 3’ ends (in yellow) that increases PCR efficiency and the quantity of product generated.

EBV:
upstream primer: 5’ - AAAATCTCGTATGTTTATCGCAG - 3’
downstream primer: 5’ - AAAATCTCGTATGTTTATCGCAG - 3’
probe: 5’ - MBG - FAM - GTACGA'TGATGCTGAGCA - NFQ - 3’

GTTAATCCGATCTGTCCAG
GCAATCGTATTACCTCTTATCGCAG

Table 1

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We made dilutions of our standard (a plasmid containing the cloned amplicon) and tested them in our assay. The EBV amplification plot is shown in Figure 1, while the amplification plot for the internal control is shown in Figure 2. The internal control Cq is unchanged over 6 logs of target concentration. The melting temperature of the probe is approximately 69°C (Figure 3). We generated a 6 log standard curve (Figure 4) with a range of 10 to 1,000,000 copies per reaction (390 to 39,000,000 copies per µL). The slope of the standard curve was -3.52 (PCR efficiency = 82%) with an R2 value of 0.997. When we compared the sensitivity of this new assay with that of the LightCycler assay currently used in our clinical lab, by testing serial 2-fold dilutions of control material, that both assays displayed approximately equal sensitivity.

To evaluate the accuracy of the assay, we tested 66 plasma and serum samples (and one whole blood) that had been previously quantitated at a major reference lab. The results of this comparison are shown in Table 1. Our Eclipse assay generated consistently higher quantitative values than those of the reference lab, by approximately 0.5 log (mean difference = 0.6 log). Regardless of the quantitative values generated, these 2 assays display good agreement when qualitative results are examined, indicating that the limits of detection are likely similar for both methods. Of the 67 samples tested, both methods were in agreement for 59 (88%). The other 9 samples (highlighted in yellow) were low positives, with an even distribution between those that went undetected at ARUP and those that went undetected at the reference lab. These results suggest that differences in the standard material may be responsible for the differences in the quantitative values generated by each assay. These differences are currently being explored.

As part of our initial validation, we tested 23 randomly selected whole blood samples that had been sent to ARUP for testing (Table 2). Interestingly, nearly 40% (9/23) were reproducibly positive by our assay. Another 3 (highlighted in yellow) gave indeterminate results, consistent with these samples being low positives, near our limit of detection.