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

ARUP LABORATORIES

An enterprise of the University of Utah and its Department of Pathology

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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 BNFR1 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. Sequestration of reagent lots and the lyophilization of control material have been shown to help maintain stability of assay performance over the period that a stored standard curve is being used. A plasmid internal control is included in the sample extraction to detect PCR inhibition and extraction failures.

MATERIALS and METHODS

Extractions:

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 Nanogen/Epoch 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 PY559, 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 (MGB). The 3' end of the EBV probe is labeled with a non-fluorescent quencher (NFQ). The internal control probe is also labeled at the 3' end with a non-fluorescent quencher. It is labeled at the 5' end with a fluorescent dye, PY559, and the MGB. The asterisks in the probe sequences indicate guanosines 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-templated, 12 nucleotide AT-rich tail added to their 5' ends (in yellow) that increases PCR efficiency and the quantity of product generated.

EBV:

upstream primer: 5' - AATAAATCATAAGTTAATCCGATCTGGTCGCA - 3'
downstream primer: 5' - AATAAATCATAAGAACCTGGTCMTCCTTTG - 3'
probe: 5' - MGB - FAM - G*TACG*AGTG*CCTG*CG*A - NFQ - 3'

→
GTTAATCCGATCTGGTCGCAGGCACTCGTACTGCTCGCTGGCAAAGGAKGACCAGGTTC
←

IC:

upstream primer: 5' - GCAATCGTATTACCTCTTATCGCAG - 3'
downstream primer: 5' - CAACCATCGTCATCGTCAGGAAAC - 3'
probe: 5' - MGB - PY559 - G*CAAAGTCCCATCGTT- NFQ - 3'

→
GCAATCGTATTACCTCTTATCGCAGCTGGTTCCTATTTGGCAAAGTCCCATCGTTTCCTGACGATGACGATGGTTG
←

We perform the assay using 10μL of extracted DNA in a 50μL total reaction volume. In addition to the target DNA, we include the following reagents:

	final conc.
EBV/IC primer mix	1x
EBV/IC probe mix	1x
$MgCl_2$	4 mM
UNG (Perkin Elmer)	0.5 U
LC FastStart Hyb Probe Mix (Roche)	1x
PCR Enhancer (Epicentre)	1x
ROX	60 nM

The final reaction concentration of the primers is 1 μM each for the EBV primers and 300 nM each for the internal control primers. Both probes are present in the reaction at a concentration of 200 nM.

The cycling conditions for amplification and detection are as follows:

1) 50°C 2 minutes
2) 95°C 8 minutes

3) 95°C 15 seconds
4) 58°C 30 seconds
5) 76°C 30 seconds
(steps 3–5: 50 cycles)

6) 95°C 15 seconds
7) 45°C 15 seconds
8) 95°C 15 seconds

All ramp rates are set to 100%, except for the final ramp which is set to 5%. Data is collected at step 4 and during the temperature ramp between steps 7 and 8.

Figure 1

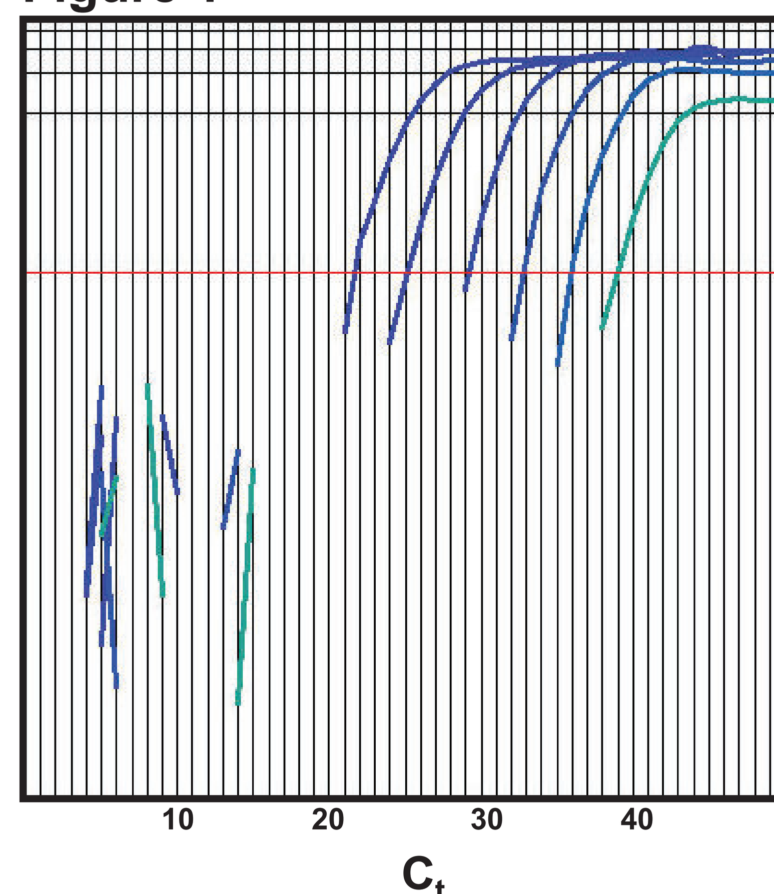


Figure 2

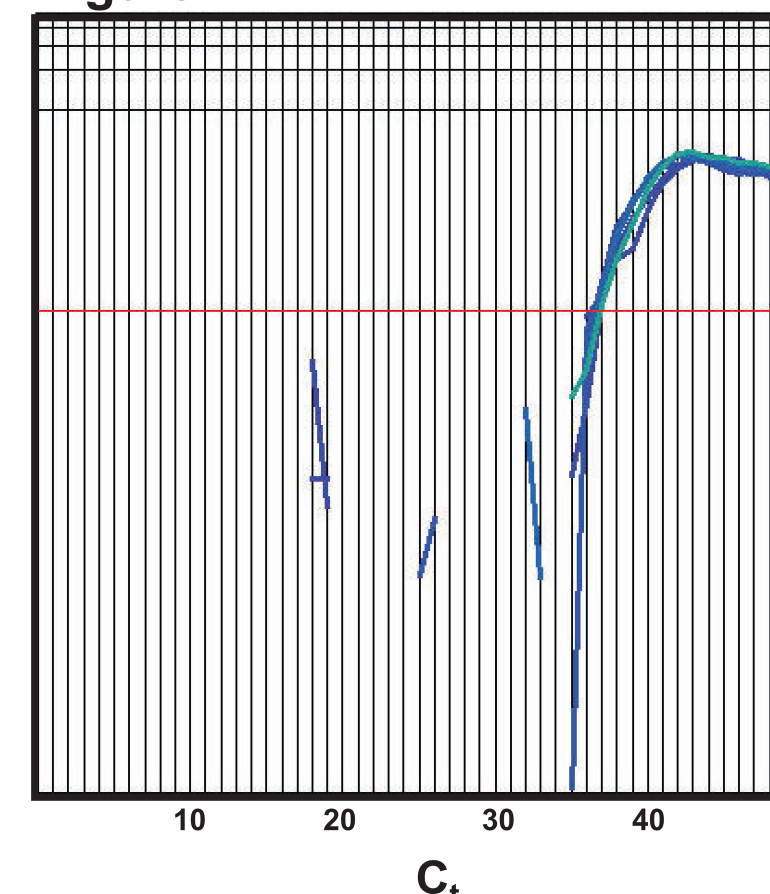


Figure 3

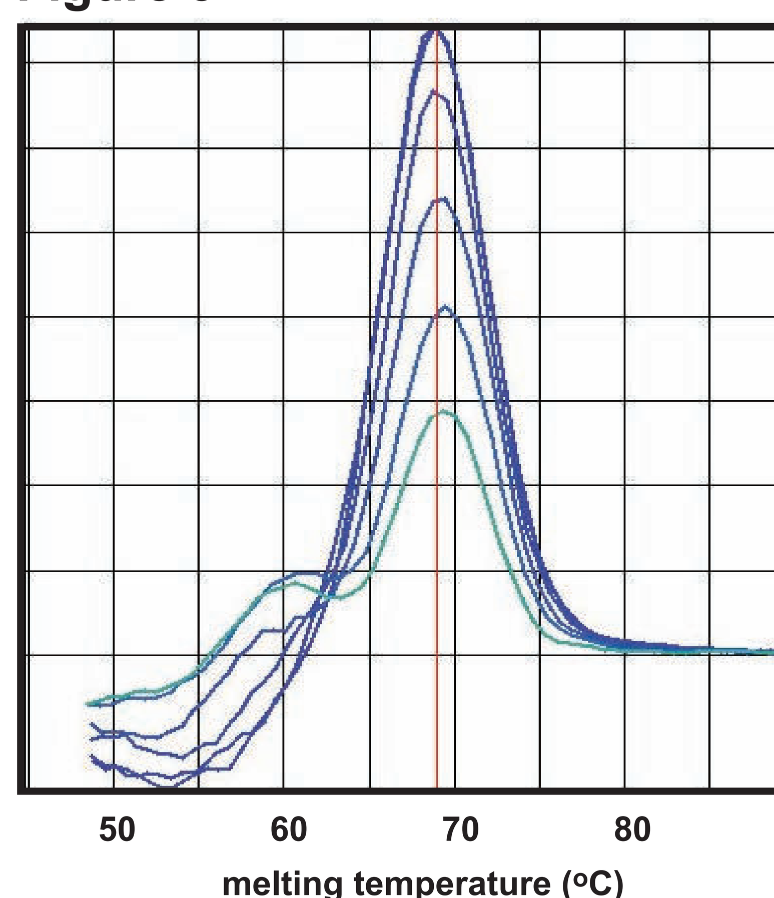
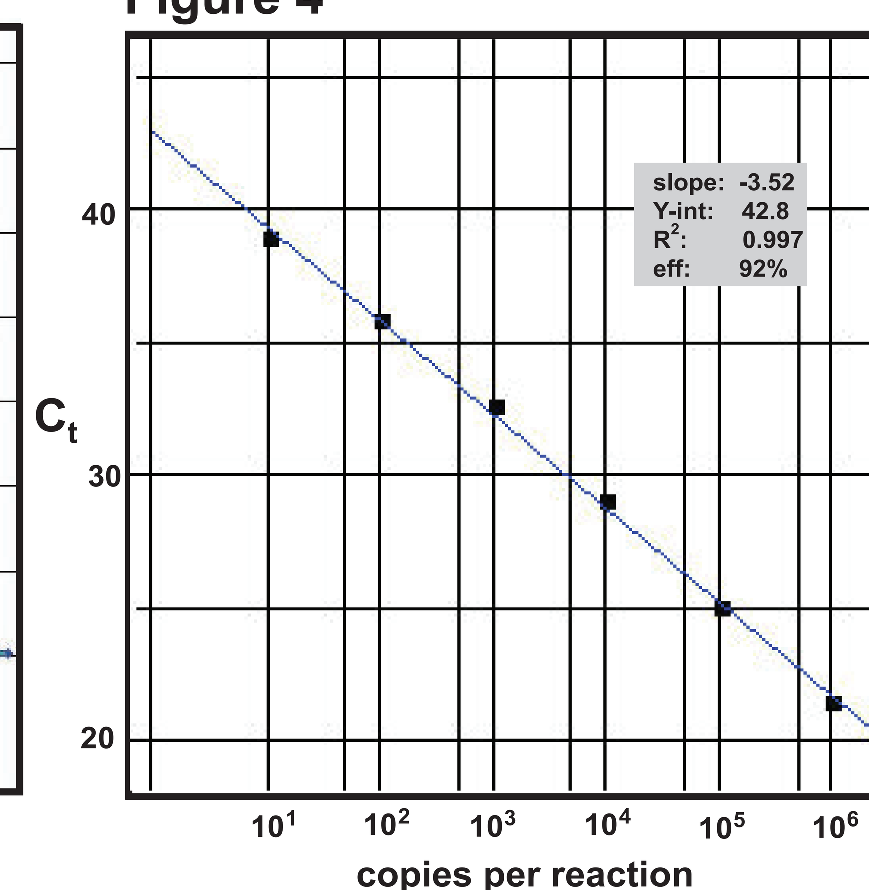


Figure 4



RESULTS

We made log 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 C_t 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 mL). The slope of the standard curve was -3.52 (PCR efficiency = 92%) 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 8 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.

Table 1

	EBV viral load (log copies/mL			
Sample	initial testing	repeat testing	reference lab result	sample type
JSEBV 101905-01	3.7	3.6	2.3	plasma
JSEBV 101905-02	4.5		3.9	plasma
JSEBV 101905-03	3.7	4.0	2.3	plasma
JSEBV 101905-04	3.7	3.3	2.8	plasma
JSEBV 101905-05	neg	2.9, neg	2.8	serum
JSEBV 101905-06	neg		<2.0	plasma
JSEBV 101905-07	2.1		2.5	plasma
JSEBV 101905-08	3.9	3.7	3.2	plasma
JSEBV 101905-09	3.4		3.0	plasma
JSEBV 101905-10	2.9	2.6	2.0	plasma
JSEBV 101905-11	2.8		2.3	plasma
JSEBV 101905-12	3.6		3.0	plasma
JSEBV 101905-13	3.6		3.3	plasma
JSEBV 101905-14	2.6		2.3	plasma
JSEBV 101905-15	neg	3.3, 2.7	2.0	serum
JSEBV 101905-16	neg		<2.0	plasma
JSEBV 101905-17	3.5	3.7	2.3	plasma
JSEBV 101905-18	3.8		3.5	plasma
JSEBV 101905-19	3.9		3.4	plasma
JSEBV 101905-20	3.4	3.7	2.7	plasma
JSEBV 101905-21	2.6	2.4	2.0	plasma
JSEBV 101905-22	3.7	3.5	3.0	plasma
JS-EBV080405-1	neg		<2.0	serum
JS-EBV080405-2	neg		<2.0	serum
JS-EBV080405-3	neg		<2.0	serum
JS-EBV080405-4	neg		<2.0	serum
JS-EBV080405-5	neg		<2.0	serum
JS-EBV080405-6	neg		<2.0	serum
JS-EBV080405-7	neg		<2.0	serum
JS-EBV080405-8	neg		<2.0	serum
JS-EBV080405-9	neg		<2.0	serum
JS-EBV080405-10	neg		<2.0	serum
JS-EBV080405-11	2.9	2.4	<2.0	serum
JS-EBV080405-12	neg		<2.0	serum
JS-EBV080405-13	2.3	neg, 2.0	<2.0	serum
JS-EBV080405-14	neg		<2.0	serum
JS-EBV080405-15	neg		<2.0	serum
JS-EBV080405-16	4.9		4.3	whole blood
JS-EBV080405-17	2.6		2.6	serum
JS-EBV080405-18	3.4	2.9	2.6	serum
JS-EBV080405-19	4.5	4.2	3.0	serum
JS-EBV080405-20	3.1	2.6	2.5	serum
JS-EBV080405-21	3.3		3.2	serum
JS-EBV080405-22	2.9	2.0	2.3	serum
JS-EBV080405-23	neg	neg	3.1	serum
JS-EBV080405-24	3.6		3.9	serum
JS-EBV080405-25	3.8	3.0	3.1	serum
JS-EBV080405-26	4.4	4.0	3.6	serum
JS-EBV080405-27	2.7	2.7	2.0	plasma
JS-EBV080405-28	2.4	2.4	<2.0	plasma
JS-EBV080405-29	3.2	2.6	2.3	plasma
JS-EBV080405-30	2.3		2.0	plasma
JS-EBV080405-31	neg		<2.0	plasma
JS-EBV080405-32	neg		<2.0	plasma
JS-EBV080405-33	neg		<2.0	plasma
JS-EBV080405-34	neg	neg	2.0	plasma
JS-EBV080405-35	3.7	3.0	2.5	plasma
JS-EBV080405-36	neg		<2.0	plasma
JS-EBV080405-37	neg		<2.0	plasma
JS-EBV080405-38	neg		<2.0	plasma
JS-EBV080405-39	3.5	3.1	2.8	plasma
JS-EBV080405-40	neg		<2.0	plasma
JS-EBV080405-41	neg		<2.0	plasma
JS-EBV080405-42	neg		<2.0	plasma
JS-EBV080405-43	neg		<2.0	plasma
JS-EBV080405-44	neg		<2.0	plasma
JS-EBV080405-45	2.7	neg	<2.0	plasma

Table 2

	EBV viral load (log copies/mL)	
Sample	initial testing	repeat testing
whole blood 1	2.7	3.4
whole blood 2	neg	neg
whole blood 3	neg	2.1
whole blood 4	neg	neg
whole blood 5	neg	neg
whole blood 6	4.9	4.5
whole blood 7	4.3	3.6
whole blood 8	neg	neg
whole blood 9	neg	neg
whole blood 10	neg	neg
whole blood 11	neg	neg
whole blood 12	neg	neg
whole blood 13	3.2	neg, 2.2
whole blood 14	2.9	3.1
whole blood 15	3.7	3.2
whole blood 16	neg	neg
whole blood 17	3.1	2.8
whole blood 18	neg	neg
whole blood 19	3.3	2.3
whole blood 20	neg	neg
whole blood 22	3.3	2.8
whole blood 23	3.1	3.0
whole blood 24	neg	2.5

CONCLUSIONS

Measuring EBV viral load in transplant patients is an important element in determining the course of treatment. This test is linear over 6 logs and has an analytical sensitivity approaching that imposed by sampling error. Use of a hybridization probe provides the opportunity to perform a melt curve analysis, which can aid in preventing false negatives due to sequence variations in the target. Accurate quantitative PCR requires accurate quantitation of standard material. The differences between results from the ARUP assay and that of our reference lab are not likely due to differences in sensitivity, but small differences in the quantitation of standards at each laboratory. This emphasizes the need for a common standard material that can be used to improve the consistency of results between labs.