Performance Characteristics of Two Real-Time PCR Assays for the Quantification of Epstein-Barr Virus DNA

Charles E. Hill, MD, PhD, Shealynn B. Harris, MD, Elizabeth E. Culler, MD, James C. Zimring, MD, PhD, Frederick S. Nolte, PhD, and Angela M. Caliendo, MD, PhD

Key Words: Epstein-Barr virus; EBV; Viral load; Posttransplantation lymphoproliferative disorder; PTLD; Real-time polymerase chain reaction

DOI: 10.1309/ABEYV2VKE6DHXAAA

Abstract

We compared the performance of a laboratory-developed 5'-nuclease real-time polymerase chain reaction assay and a commercial assay (LightCycler, Roche Diagnostics, Indianapolis, IN) for quantification of Epstein-Barr virus (EBV) DNA. Using standards provided by the manufacturer, the LightCycler assay was linear from 100 to 1 million copies per reaction. Based on dilution of a plasmid containing the amplicon, the laboratory-developed assay was linear from 22 to 45 million copies per reaction. Both assays detected 0.5 copies of genomic EBV DNA per reaction; both showed good reproducibility with coefficients of variation from 0.3% to 2.4% for the LightCycler and 1.8% to 5.1% for the laboratory-developed assay. For 31 whole blood specimens with measurable values in both assays, the viral load values obtained with the LightCycler averaged 2.3-fold higher than those obtained with the laboratory-developed assay. Testing 30 matched whole blood and plasma samples in the laboratory-developed assay showed whole blood viral load values averaged 10-fold higher than those for plasma. The LightCycler and laboratory-developed assays are sensitive and reproducible with broad linear ranges. Further clinical evaluation is needed to determine the viral load cutoff that is predictive of posttransplantation lymphoproliferative disorders.

Epstein-Barr virus (EBV)-associated posttransplantation lymphoproliferative disorders (PTLDs) are rare but severe complications following transplantation, representing a spectrum of diseases ranging from a mononucleosis-like illness to non-Hodgkin lymphoma. PTLDs result from abnormal lymphoid proliferation, generally of B-cell origin, driven by EBV transformation of lymphocytes. In transplant recipients, ineffective T-cell function resulting from immunosuppressive therapy allows EBV to replicate unchecked and ultimately allows the development of a neoplastic B-cell clone.

The incidence of PTLDs ranges from 0.8% to 20% and is dependent on many factors, including type of organ transplanted, age at transplantation, and the type of immunosuppressive therapy. Generally, PTLDs occur more frequently in children than in adults, which is likely due to EBV seronegativity; children are more likely to be EBV seronegative (49%) than adults (8%) at the time of transplantation. Seronegative adults also have an increased risk of developing PTLDs. These findings suggest that PTLDs may be more likely in patients experiencing primary EBV infection while receiving immunosuppressive agents. Other risk factors for PTLDs include the development of cytomegalovirus (CMV) disease (especially in the D+/R− CMV serogroup), HLA-mismatched T-cell depletion of the donor stem cells, the use of antilymphocytic antibodies as conditioning, and treatment of graft-vs-host disease.

The clinical manifestations of PTLD are diverse with regard to symptoms, involved site, and severity. Although history, physical examination, and imaging may provide diagnostic clues, a definitive diagnosis requires tissue biopsy and demonstration of EBV by immunohistochemical staining and/or in situ hybridization.
There is interest in determining whether molecular assays could be useful for identifying patients at high risk of developing PTLDs, allowing early intervention in the hope of decreasing morbidity and mortality. Several studies have supported this approach by showing that a high level of EBV DNA in lymphocytes is associated with the development of PTLDs. A study of allogeneic stem cell transplant recipients showed that the median viral load in whole blood of patients with PTLDs was 1.6 × 10^6 copies/µg DNA compared with 4 × 10^3 copies/µg DNA in patients without PTLDs. Another study used a quantitative EBV DNA polymerase chain reaction (PCR) assay to frequently monitor stem cell transplant recipients at high risk of developing PTLDs. When the viral load in plasma exceeded 1,000 genome equivalents per milliliter, patients received preemptive therapy with rituximab. This approach improved patient outcomes compared with historic control subjects, with 2 of 17 patients in the preemptive therapy group developing PTLDs compared with 10 of 26 historic control subjects. Based on these studies, it seems that monitoring patients at risk for PTLDs using an EBV viral load assay has merit, although the threshold viral load for defining people at risk of PTLDs will be influenced by the specific assay and specimen type.

There is no US Food and Drug Administration–cleared or “gold standard” assay available for the quantification of EBV DNA, so laboratories performing this type of testing rely on laboratory-developed assays or one of the few commercial assays for EBV quantification. The performance of these assays can be reliable in experienced laboratories; however, depending on assay design, the viral load values between assays may not always agree. Also, unlike hepatitis C virus, there is no international reference material against which to calibrate assays, so results may not be comparable between laboratories. Thus, it is difficult to establish clinically relevant viral load cutoff values that can be applied broadly in clinical practice. In the present study, using clinical specimens, we compared the sensitivity, linear range, and agreement of viral load values between 2 quantitative real-time EBV PCR assays, a laboratory-developed assay and the LightCycler EBV Quantification Kit (Roche Diagnostics, Indianapolis, IN).

**Materials and Methods**

**Specimens and Reagents**

Whole blood specimens were obtained from patients being treated at Children’s Healthcare of Atlanta (Atlanta, GA) and were sent to a referral laboratory for EBV PCR testing as part of routine clinical care. We collected any remaining specimen that was available after clinical testing. The study was approved by the institutional review boards of Emory University Hospital, Atlanta, and Children’s Healthcare of Atlanta (No. 247-2001).

Specimens were obtained primarily in acid citrate dextrose tubes; however, a portion was anticoagulated with EDTA (reflecting the specimen acceptability requirements of the reference laboratory). An aliquot of the whole blood (200 µL) was processed within 24 hours of receipt with the QIAGEN DNA Mini kit using the manufacturer’s whole blood protocol (QIAGEN, Valencia, CA), eluting in 50 or 200 µL, and the extracted nucleic acid was stored at –80°C until testing. If there was adequate specimen, a second aliquot of whole blood was centrifuged at 1,500g for 10 minutes, and the plasma fraction was stored at –80°C. At the time of testing, nucleic acid was extracted from the plasma samples (200 µL) by using the MagNA Pure LC System (total nucleic acid kit, Roche Diagnostics) and eluted in 65 µL (minimum elution volume). When comparing viral load values between the LightCycler and laboratory-developed assays, the aliquot of nucleic acid derived from the whole blood specimen was tested in both assays. Also, viral load values in plasma and whole blood were compared using samples that were derived from the same clinical specimen.

As a reference quantification standard, a plasmid was constructed containing the sequence of the amplicon used in the laboratory-developed assay. This was done to allow a consistent source of DNA to which a copy number could be assigned. Primers were designed to encompass the amplicon sequence plus 6 base pairs upstream and downstream of the binding sites for the primers used for quantification. The primer sequences were as follows: forward, 5’-GCG TTT ACG TAA GCC AGA CAG CAG CC-3’ and reverse, 5’-CTC ACA TTT GTG TGG ACT CCT CGC G-3’ (additional sequences added compared with quantification primers are underlined).

The amplicon was T/A ligated into the pGEM-T-EASY vector (Promega, Madison, WI) according to the manufacturer’s instructions. The ligation reaction was transformed into chemically competent DH5α *Escherichia coli* by heat shock according to the manufacturer’s protocol (One Shot MAX Efficiency DH5-T1R Cells, Invitrogen, Carlsbad, CA). Blue-white screening using ampicillin/X-gal agar plates was used to identify colonies that contained plasmids with an insert. After confirmation of the insert by size, the plasmid was purified by using the QIAGEN Midi prep system. The concentration of the plasmid was determined using both A260 values and fluorescence signal with picogreen prep system. The concentration of the plasmid was determined using both A260 values and fluorescence signal with picogreen prep system. The concentration of the plasmid was determined using both A260 values and fluorescence signal with picogreen prep system. The concentration of the plasmid was determined using both A260 values and fluorescence signal with picogreen prep system. The concentration of the plasmid was determined using both A260 values and fluorescence signal with picogreen prep system. The concentration of the plasmid was determined using both A260 values and fluorescence signal with picogreen prep system. The concentration of the plasmid was determined using both A260 values and fluorescence signal with picogreen prep system. The concentration of the plasmid was determined using both A260 values and fluorescence signal with picogreen prep system. The concentration of the plasmid was determined using both A260 values and fluorescence signal with picogreen prep system.
EBV Real-Time PCR Assays

Testing using the LightCycler EBV Quantification Kit, which targets the LMP 2 gene, (Roche Diagnostics, for research use only) was performed following the manufacturer’s protocol (http://www.roche-diagnostics.com/ba_rmd/rmd_products_microbiology_08.html). The kit included amplification and detection reagents, 5 EBV DNA standards, and an optional internal control (which was not used in this study). The EBV Quantification Kit tests 5 µL of extracted DNA per reaction.

The laboratory-developed assay amplifies a 114-base-pair region of the BAM HI-W region, a repetitive, highly conserved region within the EBV genome. The primer and probe sequences were as follows: BAM HI-W forward, 5’-ACG TAA GCC AGA CAG CAG CC-3’; BAM HI-W reverse, 5’- TTT GTG TGG ACT CCT GGC G-3’; probe, 5’-FAM TCC TGC AGC TAT TTC TGG TCG CAT CA TAMRA-3’. The 50-µL PCR reaction contained 25 µL of 2X TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, manufactured by Roche, Branchburg, NJ), 5 µL of each primer (final concentration, 300 nmol/L), 5 µL of probe (final concentration, 200 nmol/L), and 5 µL of sample DNA.

The samples were amplified in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the following thermal cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. For both assays, the concentration of EBV DNA was determined based on a 5-point standard curve, and positive and negative control samples were included in all runs. The copy number of BAM HI-W obtained with the laboratory-developed assay was divided by 11 to take into account that the amplified sequence was repeated 11 times in the EBV genome, yielding the number of EBV genomes per milliliter.

Statistics

Data were log_{10} transformed before analysis. Descriptive statistics and regression line equations were calculated with the analysis tool pack of Microsoft Excel 2000 (Microsoft, Redmond, WA). Agreement between viral load values was assessed by the method of Bland and Altman.

Results

Representative standard curves for the LightCycler and laboratory-developed assays are shown in Figure 1. Using the standards provided by the manufacturer, the LightCycler assay was linear from 100 to 1 million copies per reaction (2.0-6.0 log_{10} copies per reaction). The linear range of the laboratory-developed assay was assessed using a plasmid containing the amplicon. In Figure 1B, the linear range is shown as 45 to 45 million copies per reaction (1.7 to 7.7 log_{10} copies per reaction). Further analysis using serial 2-fold dilutions showed the laboratory-developed assay was linear down to 22 copies per reaction (1.3 log_{10} copies per reaction).

The reproducibility of each real-time PCR assay was evaluated by testing multiple replicates of the standard control material. For the LightCycler assay, single replicates of the standards were run on 7 different days. For the laboratory-developed assay, duplicates of the standards were run on 7 different days. The mean, SD, and coefficient of variation (CV) of the cycle threshold (laboratory-developed assay) and crossing point (LightCycler assay) values are shown in Table 1.
Based on the data, both assays seem to have good interassay reproducibility, with CVs ranging from 0.8% to 2.7%.

Because the assays used different standard curve material, genomic EBV DNA was used to compare the limit of detection between the 2 assays. The LightCycler and laboratory-developed assays detected all 6 replicates of specimens containing 50, 5, and 0.5 copies of EBV DNA per reaction. In this analysis, the LightCycler assay seems to have better reproducibility than the laboratory-developed assay. For concentrations in which 100% of replicates were detected, the CV ranged from 0.3% to 1.1% for the LightCycler assay, compared with 3.6% to 5.1% for the laboratory-developed assay.

We tested 50 whole blood specimens in the LightCycler and the laboratory-developed assays: 31 specimens were positive in both assays, 13 were negative in both assays, and 6 were positive in the LightCycler assay and negative in the laboratory-developed assay. The mean viral load for the discrepant specimens was $3.2 \log_{10}$ copies/mL (range, 2.85-3.53 log$_{10}$ copies/mL).

When testing clinical specimens, the LightCycler assay seemed to be more sensitive than the laboratory-developed test, which was not consistent with our findings testing genomic EBV DNA. However, samples of extracted nucleic acid initially were tested in the LightCycler assay and then stored for several additional months before they were tested in the laboratory-developed assay, so there may have been some DNA degradation before testing in the laboratory-developed assay.

To further assess the role of specimen integrity, DNA was extracted from 6 specimens and tested (in duplicate) the same day in the LightCycler and the laboratory-developed assays. These specimens were whole blood specimens from transplant recipients and spanned the dynamic range. The mean difference in viral load measurements (LightCycler minus laboratory-developed assay) was $-0.20 \log_{10}$ copies/mL (SD, 1.01 log$_{10}$ copies/mL; median, $-0.51 \log_{10}$ copies/mL; range, 1.72 to $-1.03 \log_{10}$ copies/mL). One of the specimens, which measured 52 copies/mL on the LightCycler assay, was undetectable by the laboratory-developed assay.

For the 31 specimens with a detectable EBV DNA in both assays, the agreement of the viral load values between the 2 assays is shown in Figure 2. The mean difference in the viral load values (LightCycler minus laboratory-developed assay) was $0.36 \log_{10}$ copies/mL with an SD of 0.57 log$_{10}$ copies/mL (median, 0.26 log$_{10}$ copies/mL; range, $-0.69$ to 2.24 log$_{10}$ copies/mL). On average, the viral load values with the LightCycler were 2.3-fold higher than those obtained with the laboratory-developed assay. These differences in viral load values may be due to assay design, the standard material used for quantification, or DNA degradation before testing in the laboratory-developed assay. To assess differences in viral load values between specimen types, 30 matched whole blood and plasma specimens were tested in the laboratory-developed assay. On average, the quantity of DNA in whole blood specimens was 0.96 log$_{10}$ higher than that measured in plasma (median difference, 1.14 log$_{10}$ copies/mL; range, $-0.58$ to 2.98 log$_{10}$ copies/mL).

### Table 1

<table>
<thead>
<tr>
<th>Concentration (log$_{10}$)</th>
<th>Mean CP</th>
<th>SD</th>
<th>CV (%)</th>
<th>Mean CT</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>34.2</td>
<td>0.5</td>
<td>1.2</td>
<td>1.7</td>
<td>0.7</td>
<td>1.9</td>
</tr>
<tr>
<td>3.0</td>
<td>30.6</td>
<td>0.5</td>
<td>1.6</td>
<td>2.7</td>
<td>0.6</td>
<td>1.8</td>
</tr>
<tr>
<td>4.0</td>
<td>27.3</td>
<td>0.5</td>
<td>1.8</td>
<td>3.7</td>
<td>0.7</td>
<td>2.1</td>
</tr>
<tr>
<td>5.0</td>
<td>24.0</td>
<td>0.2</td>
<td>0.8</td>
<td>4.7</td>
<td>0.7</td>
<td>2.7</td>
</tr>
<tr>
<td>6.0</td>
<td>20.7</td>
<td>0.5</td>
<td>2.4</td>
<td>5.7</td>
<td>0.5</td>
<td>2.2</td>
</tr>
</tbody>
</table>

CP, crossing point; CT, cycle threshold; CV, coefficient of variation.

### Table 2

#### Analytic Sensitivity of the LightCycler and Laboratory-Developed Assays

<table>
<thead>
<tr>
<th>EBV DNA Copies per Reaction</th>
<th>No. Positive/No. Tested</th>
<th>Mean CP (SD)</th>
<th>CV (%)</th>
<th>No. Positive/No. Tested</th>
<th>Mean CT (SD)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>4/6</td>
<td>38.6 (1.9)</td>
<td>4.9</td>
<td>5/6</td>
<td>37.2 (1.7)</td>
<td>4.6</td>
</tr>
<tr>
<td>0.5</td>
<td>6/6</td>
<td>35.0 (0.1)</td>
<td>0.3</td>
<td>6/6</td>
<td>33.7 (1.2)</td>
<td>3.6</td>
</tr>
<tr>
<td>5</td>
<td>6/6</td>
<td>32.5 (0.3)</td>
<td>0.9</td>
<td>6/6</td>
<td>31.2 (1.6)</td>
<td>5.1</td>
</tr>
<tr>
<td>50</td>
<td>6/6</td>
<td>22.4 (0.3)</td>
<td>1.1</td>
<td>6/6</td>
<td>26.6 (1.3)</td>
<td>4.9</td>
</tr>
</tbody>
</table>

CP, crossing point; CT, cycle threshold; CV, coefficient of variation; EBV, Epstein-Barr virus.
There is not a commercial source for EBV standard material, so we were unable to assess the accuracy of the 2 assays. However, our laboratory participated in the Quality Control for Molecular Diagnostics proficiency challenge, which is jointly sponsored by the European Society for Clinical Virology and the European Society for Clinical Microbiology and Infectious Diseases (Glasgow, Scotland). The 8 panel members were constructed by diluting electron microscope–counted viral particles in plasma. The samples were tested in the in-laboratory assay only, and the results were in close agreement with the expected concentration. Table 3.

During our evaluation of the 2 real-time EBV PCR assays, we had specimens from a few patients in whom biopsy- or autopsy-confirmed PTLDs developed. The diagnosis of PTLD in these patients was established by morphologic assessment, immunohistochemical analysis, and/or in situ hybridization for EBV on biopsy or autopsy material. One case occurred in an adult and 3 in children. The viral load values (laboratory-developed assay) at or near the time of the diagnosis of PTLDs ranged from $2.1 \times 10^5$ to $2.1 \times 10^6$ copies/mL ($5.3$ to $6.3 \log_{10}$ copies/mL). In several of the patients who were monitored, viral load levels decreased in response to reductions in immunosuppressive therapy.

A cost analysis of the LightCycler and laboratory-developed assays was conducted in which the cost of labor, reagents, disposables, controls, and standards was determined per reportable result, assuming specimens were run as a single determination. Based on our volume of 1,000 tests per year with 2 runs per week, the cost of the laboratory-developed assay, including the royalty payment and MagnaPure extraction, was approximately $20 per reportable result compared with $108 for the LightCycler assay. Approximately 75% of the cost of the LightCycler assay was for the PCR reagents; laboratories with a greater volume of tests can expect to have a lower reagent cost.

Discussion

Monitoring patients for EBV viral load to assess the risk of developing PTLDs is used with increasing regularity in transplant centers. Because there are no Food and Drug Administration–cleared assays for the quantification of EBV DNA, laboratories must rely on analyte-specific reagents or laboratory-developed assays. In the present study, we assessed the performance of 2 such assays. To our knowledge, this is the first study assessing the performance of the LightCycler assay. The LightCycler and the laboratory-developed assays were able to detect as few as 0.5 copies of genomic EBV DNA per reaction. According to the manufacturer, the viral copy number of the purified genomic DNA used in the study was determined by hybridization to radiolabeled probes and compared with a calibrated standard fragment. The DNA copy number may vary depending on the method of quantification, which may explain why the assays were able to detect less than 1 copy of genomic DNA per reaction.

The laboratory-developed assay was linear over 6 log_{10} and the LightCycler assay was linear over at least 4 log_{10} (standard material with higher viral load values was not provided). In addition, both assays showed good reproducibility with CVs of the crossing point and cycle threshold values ranging from less than 1.0% to 5.1%. Overall, it seems that the LightCycler assay is slightly more reproducible than the laboratory-developed assay. This may reflect subtle differences in assay design and/or sensitivity because the LightCycler standards were detected at somewhat lower cycle numbers.

When comparing viral load values between the 2 assays using clinical specimens, the levels of EBV DNA measured in the LightCycler assay were, on average, 2.3-fold higher than those seen with the laboratory-developed assay. This is remarkably close agreement considering that the assays target different regions of the EBV genome and use different input
specimen volumes and different standard curve material. Without an international reference standard, we were unable to assess the accuracy of the 2 assays. However, the viral load values obtained with the laboratory-developed assay were in close agreement with proficiency samples of diluted electron microscope–counted viral particles. Based on this panel, the laboratory-developed assay seems to have a lower limit of sensitivity of approximately 300 copies/mL (2.5 log_{10} copies/mL), which is consistent with what we observed with the plasmid standard (22 copies per reaction or 140 copies/mL).

A limitation of the study is that specimens were stored for a longer period before testing in the laboratory-developed assay, which may have affected the agreement between the assays. Based on a limited number of specimens that were extracted and tested the same day, the mean difference in viral load between the assays for fresh vs stored specimens was not significantly different (mean ± SD, –0.20 ± 1.01 log_{10} copies/mL vs 0.36 ± 0.57 log_{10} copies/mL, respectively). Although there may have been some degradation of the DNA during storage, it seems that this contribution to variability may be within the error of the measure.

There is no consensus regarding the appropriate specimen type for EBV viral load testing. The laboratory-developed assay was designed to test whole blood and plasma specimens, with the expectation that whole blood values would be higher than plasma values because EBV is a cell-associated virus. In fact, we observed that viral load values in whole blood samples were on average 0.9 log_{10} higher than those observed in matched plasma samples. However, for adults, there may be some value in testing plasma specimens. With more than 95% of adults seropositive for EBV at the time of transplantation, asymptomatic reactivation of EBV that does not progress to a PTLD is common. Testing plasma rather than whole blood in this population may aid in distinguishing patients at risk for PTLDs from those with asymptomatic reactivation. Recent studies have shown the clinical usefulness of monitoring EBV viral load in plasma specimens to assess the risk of developing PTLD. Based on what has been observed for CMV, it is likely that the threshold viral load value that correlates with the risk of developing a PTLD will need to be established for each specimen type and, possibly, each assay.

Pediatric patients present a different challenge because many children are seronegative at the time of transplantation, putting them at higher risk for PTLDs. As a result, there is interest in detecting EBV DNA as early as possible to mark the onset of primary infection because antibody production may not be a reliable indicator of infection after transplantation. Early in the infection, the virus may be underquantified in plasma as a large fraction of virus is cell-associated. For this reason, EBV viral load testing in our pediatric transplant recipients is done using whole blood samples. It must be noted, however, that there is no established clinical benefit of using blood instead of plasma in this patient population.

The cost analysis of the 2 assays showed that based on our volume of 1,000 tests per year and 2 runs per week, the laboratory-developed assay was much less expensive per reportable result ($20) than the LightCycler assay ($108). However, the cost of the LightCycler assay would be expected to decrease with a higher testing volume or if testing were done once per week. Although more costly, there are advantages to the LightCycler assay. The material for the standard curve is provided in the kit, the time for amplification is approximately 1 hour compared with 2.5 for the laboratory-developed assay, and it allows for standardization of testing between laboratories. Advantages to the laboratory-developed assay are the use of standard reagents and thermal cycle parameters, which allows the flexibility of testing multiple analytes on the same plate. However, the manufacture and quality control of the quantification standard (plasmid) adds additional cost and requires considerable time and labor. Available equipment and technical expertise are other factors to consider when deciding which assay to use for clinical testing.

We have shown that the LightCycler and the laboratory-developed assays are sensitive, with a 4 to 6 log linear range and good reproducibility for clinical testing. Additional clinical experience is needed to determine what viral load cutoff is predictive of developing PTLDs in pediatric and adult patients. We are now observing patients prospectively to determine the viral load value that may predict the development of PTLDs and, thus, trigger a reduction in immunosuppressive therapy or initiation of antiviral or anti-CD20 therapy.

**References**


