Validation of an Epstein-Barr Viral Load Assay Using the QIAGEN Artus EBV TM PCR Analyte-Specific Reagent

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Key Words: Epstein-Barr virus; Viral load; Analyte-specific reagent; QIAGEN Artus

DOI: 10.1309/AJCP04IZAMPISEWQ

Abstract

We describe the validation of a test for the quantification of Epstein-Barr virus (EBV) DNA (viral load) using the Artus EBV TM PCR analyte-specific reagent (ASR; QIAGEN Hamburg, Hamburg, Germany). A dilution series demonstrated a limit of detection of 2.25 log_{10} copies/mL (>95% positivity rate). The limit of quantification was 3.90 log_{10} copies/mL based on an SD of less than 0.15. The assay was linear from 2.17 to 6.2 log_{10} copies/mL. Low (3.70 log_{10} copies/mL) and high (5.40 log_{10} copies/mL) patient samples had coefficients of variation (CVs) of 2.0% and 1.4%, respectively. The cycle thresholds of 4 points used to generate the standard curve had CVs ranging from 0.8% to 1.6%. A comparison of 35 matched samples showed a small positive bias (0.35 log_{10} copies/mL) for the Artus ASR relative to a laboratory-developed EBV viral load assay targeting the BamHI-W region of the EBV genome.

Epstein-Barr virus (EBV) is a common γ herpesvirus that causes acute symptomatology in neonates and immunocompromised adults.1 Chronic EBV infection has been linked to the development of a number of lymphomas, including Burkitt lymphoma, extranodal nasal type NK/T-cell lymphoma, angioimmunoblastic T-cell lymphoma, Hodgkin lymphoma, posttransplantation and immunodeficiency-related lymphoproliferative disorders, and HIV-related lymphomas, as well as some solid tumors. In the setting of solid organ or stem cell transplantation, quantification of EBV DNA has prognostic value in the management of posttransplantation lymphoproliferative disorders.2-5 At present, there is no US Food and Drug Administration–approved test for EBV viral load quantification; therefore, most clinical testing for this application relies on laboratory-developed tests. Moreover, the lack of an international standard for EBV makes it difficult to standardize EBV measurement across different platforms and laboratories.6

To better standardize our quantitative results, we compared a previously reported,7 laboratory-developed assay for EBV with a new assay designed using the Artus EBV TM PCR analyte-specific reagent (ASR; QIAGEN Hamburg, Hamburg, Germany). In addition to the benefit of using commercially prepared reagents, such as improved quality control from good manufacturing practices, the Artus method incorporates a second, heterologous amplification system, which serves as an internal control to verify the robustness of the assay and identify specimens with polymerase chain reaction (PCR) inhibition or extraction failure. We determined the limit of detection (LOD), limit of quantification (LOQ), linearity, and reproducibility of the laboratory-developed assay using the Artus ASR. By using patient samples previously tested...
in our laboratory-developed assay, the agreement between a laboratory-developed assay and the Artus ASR was assessed. The performance of the Artus ASR was also evaluated using proficiency samples that had been previously tested using a laboratory-developed assay.

Materials and Methods

Specimens

Patient specimens (whole blood anticoagulated in EDTA) were sent to the Emory University Hospital Molecular Diagnostics Laboratory (Atlanta, GA) for routine EBV viral load testing using a laboratory-developed assay. Residual whole blood was stored at 4°C until tested with the Artus ASR. Testing with the ASR was completed within 1 week of storage. EBV DNA is stable in whole blood stored up to 7 days at 4°C (data not shown). This protocol was approved by the Emory University Institutional Review Board (protocol 247-2001).

Laboratory-Developed EBV Viral Load Test

The laboratory-developed method was performed as previously described. Briefly, nucleic acid was extracted from 200 µL of whole blood on the Roche MagNA Pure LC automated isolation system using the Roche MagNA Pure LC Total Nucleic Acid Isolation kit (Roche Diagnostics, Indianapolis, IN). The DNA was eluted into a final volume of 50 µL, of which 5 µL was added to the master mix containing primers, probes, Taq polymerase, magnesium chloride, and buffers. The EBV viral load was then measured by quantitative real-time polymerase chain reaction (Q-PCR) using primers that amplify the conserved BamH1-W region of the EBV genome.

QIAGEN Artus EBV TM ASR

Nucleic acid extraction was performed as described above with the addition of the internal control target to the lysis buffer of the extraction kit (5 µL internal control for each 300 µL of lysis buffer). The ASR contains primers and probes that target the conserved Epstein-Barr nuclear antigen 1 (EBNA-1) region, which were labeled with the FAM fluorophore. The internal standard ASR contains primers and probes with a proprietary target, labeled with a VIC fluorophore. The internal standard ASR contains primers and probes with a proprietary target, labeled with a VIC fluorophore. Five microliters of extracted nucleic acid was added to 45 µL of master mix, which contained the 2 primer and probe sets, nucleotides, polymerase, and magnesium. The 2 primer sets were amplified by Q-PCR using the ABI Prism 7500 or 7000 instrument (Applied Biosystems, Foster City, CA), using the following thermal cycler conditions: 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 55°C for 1 minute. The cycle threshold (Ct) for EBNA-1 and the internal control was calculated automatically by the system software, and the resulting Ct was used to quantify the EBV copy number by interpolation against a standard curve composed of a series of 4 quantitation standards (QS1-QS4: 50, 500, 5,000, and 50,000 copies/µL; QIAGEN). QS values are labeled as copies/µL. To adjust this copy number to copies/mL for patient samples, a conversion factor was used. This conversion factor was derived from the following equation:

\[
\text{Result (copies/mL)} = \text{Result (copies/µL)} \times \text{elution volume (50 µL/sample volume (0.2 mL))} = \text{Result (copies/µL)} \times 250
\]

The individual patient viral load was calculated from the slope and intercept of the standard curve as follows:

\[
\log_{10} \text{Patient EBV Concentration (log}_{10}\text{copies/mL)} = \frac{\text{Patient (Ct)} - \text{Intercept (Ct)}}{\text{Slope}}
\]

Study Design

LOD, LOQ, and Linearity

A cell line containing 2 copies of the EBV genome per cell (Namalwa Burkitt lymphoma cell line, ATCC CRL-1432) at an approximate cell density of 10^7 cells/mL was obtained from the American Type Culture Collection (Manassas, VA). This cell line was serially diluted in whole blood that was determined to be negative for EBV using the ASR. We tested 8 to 16 replicates of each dilution using the ASR. The LOD was defined as the lowest dilution with greater than 95% positivity rate for all replicates. The LOQ was defined as the lowest dilution with an SD of less than 0.15 log_{10} copies/mL. An SD of 0.15 log_{10} copies/mL was chosen to ensure that a laboratory could maintain the precision required to have 90% power to detect a 5-fold (0.69 log_{10}) difference in copy number between 2 samples in the same run. The linear range of the assay was determined by linear least-squares regression using Microsoft Excel 2003 (Microsoft, Redmond, WA) through the mean value for each dilution. The maximum range with an R^2 value of greater than 0.99 was then determined.

Reproducibility

Seven replicates each of patient samples with high (5.40 log_{10} copies/mL) and low (3.70 log_{10} copies/mL) copy numbers were tested in 5 different runs. The within-run and between-run precision coefficients of variation (CVs) were calculated based on log_{10} copies/mL results for both patient samples.

Agreement

For the study, 61 patient samples were tested with the laboratory-developed assay and the ASR. Agreement between the laboratory-developed assay and ASR was determined by
using a Bland-Altman\textsuperscript{10} plot of all samples with values in both assays (35 samples). The overall bias between the laboratory-developed assay and ASR was calculated from this plot using GraphPad Prism 5 Software (GraphPad Software, San Diego, CA). In addition, using proficiency samples provided by the American Society of Transplantation (AST, Mt Laurel, NJ) and Quality Control for Molecular Diagnostics (QCMD, Glasgow, Scotland), we determined results obtained from both assays.

**Results**

**Limits of Detection and Quantification**

The LOD of the ASR was 2.25 log\textsubscript{10} copies/mL (180 copies/mL) Table I, which was equivalent to 1.30 log\textsubscript{10} copies/mL (20 copies/mL) using the Namalwa cell line. The LOQ based on an SD of 0.15 log\textsubscript{10} copies/mL or less was 3.90 log\textsubscript{10} copies/mL (Table 1). Of note, the SDs at 3.53 log\textsubscript{10} copies/mL and 2.89 log\textsubscript{10} copies/mL were 0.18 and 0.17 log\textsubscript{10} copies/mL, respectively.

**Linearity and Reproducibility**

The assay was linear from 2.17 to 6.21 log\textsubscript{10} copies/mL Figure 1, with an $R^2$ value of 0.9925 ($y = 1.0119x - 0.1184$). Reproducibility was assessed by testing 7 replicates of low and high viral load patient samples and 6 replicates of the QSs. The low patient sample had a mean (SD, CV) value of 3.70 log\textsubscript{10} copies/mL (0.07, 2.0%). The high patient sample had a mean (SD, CV) value of 5.40 log\textsubscript{10} copies/mL (0.07, 1.4%). The QSs had mean Ct (SD, CV) values as follows: QS1, 21.8 (0.4, 1.6%); QS2, 25.1 (0.4, 1.5%); QS3, 28.5 (0.2, 0.8%); and QS4, 31.8 (0.3, 1.0%).

**Agreement**

Of the 61 patient samples tested by the laboratory-developed method and the ASR, 35 were positive in both assays. Of the 6 samples that were positive in the Artus assay but not in the laboratory-developed assay, the average copy number was 1.62 log\textsubscript{10} copies/mL, with a range of 1.41 to 2.13 log\textsubscript{10} copies/mL. There was only 1 sample that was positive in the laboratory-developed assay but negative in the Artus assay, with a value of 3.23 log\textsubscript{10} copies/mL. The remaining 19 samples were negative by both assays. The 35 samples that were positive in both assays were plotted in a standard Bland-Altman\textsuperscript{10} plot Figure 2. The mean difference between

**Table I**

<table>
<thead>
<tr>
<th>EBV Cell Line (log\textsubscript{10} copies/mL)</th>
<th>Mean Artus (log\textsubscript{10} copies/mL)</th>
<th>log\textsubscript{10} Artus SD</th>
<th>log\textsubscript{10} Artus CV</th>
<th>No. Tested/No. Positive (% Positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.30</td>
<td>6.21</td>
<td>0.07</td>
<td>0.01</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>5.30</td>
<td>5.23</td>
<td>0.11</td>
<td>0.02</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>4.30</td>
<td>4.44</td>
<td>0.07</td>
<td>0.01</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>4.00</td>
<td>3.90</td>
<td>0.15</td>
<td>0.04</td>
<td>12/12 (100)</td>
</tr>
<tr>
<td>3.70</td>
<td>3.53</td>
<td>0.18</td>
<td>0.05</td>
<td>12/12 (100)</td>
</tr>
<tr>
<td>3.00</td>
<td>2.89</td>
<td>0.17</td>
<td>0.06</td>
<td>12/12 (100)</td>
</tr>
<tr>
<td>2.70</td>
<td>2.76</td>
<td>0.24</td>
<td>0.09</td>
<td>16/16 (100)</td>
</tr>
<tr>
<td>2.40</td>
<td>2.17</td>
<td>0.39</td>
<td>0.02</td>
<td>16/16 (100)</td>
</tr>
<tr>
<td>2.00</td>
<td>2.44</td>
<td>0.33</td>
<td>0.14</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>1.30</td>
<td>2.25</td>
<td>0.48</td>
<td>0.21</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>0.60</td>
<td>1.60</td>
<td>1.06</td>
<td>0.66</td>
<td>6/8 (75)</td>
</tr>
<tr>
<td>−0.10</td>
<td>1.17</td>
<td>1.02</td>
<td>0.87</td>
<td>5/8 (63)</td>
</tr>
</tbody>
</table>

CV, coefficient of variation; EBV, Epstein-Barr virus.

* The expected copy number based on the approximate initial number of Namalwa cells (ATCC CRL-1432), the mean measured copy number using the Artus assay, SD, CV, and the number and percentage of positive replicates for each dilution of the Namalwa cell line in EBV—whole blood were tabulated.
the 2 assays (bias) was $-0.35 \log_{10}$ copies/mL (2.2-fold) with 95% confidence intervals of $-1.40$ and $0.69$, indicating that on average, the Artus ASR yielded viral load values that were 2.2-fold higher than the values obtained with the laboratory-developed assay. To further assess the agreement between the 2 tests, proficiency samples obtained from QCMD and AST were tested in both assays \textbf{Table 2}. For both proficiency panels, the values obtained with the 2 assays were in close agreement; the largest difference for any sample was 0.43 log$_{10}$ copies/mL (2.7-fold).

\section*{Discussion}

Quantitative testing for EBV has important clinical usefulness for monitoring immunocompromised patients, especially in the setting of posttransplantation lymphoproliferative disease. To our knowledge, this is the first study validating the use of the Artus EBV TM ASR. This study was important in that it defined an assay method that was based on standardized components, which is necessary to impose a degree of reproducibility on assays across different laboratories. Because there is no international standard material or method to quantify EBV viral load, laboratories have relied on the consistency of their internally developed assays for a measure of reproducibility. However, this substitutes precision for accuracy and is not generalizable to other methods.\textsuperscript{6} This ASR offers laboratories the ability to generate results for this analyte that would be generally comparable. The use of this ASR provides some standardization for laboratories, although variability could still result from the use of different nucleic acid extraction methods and amplification platforms.\textsuperscript{6,11} The LOD for the laboratory-developed test and ASR were comparable, ie, 100 to 300 copies/mL. Although there is no accepted standard for LOQ for EBV, we have determined

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|}
\hline
 & Consensus, log$_{10}$ copies/mL & Laboratory-Developed Assay, log$_{10}$ copies/mL & Artus Assay, log$_{10}$ copies/mL \\
\hline
\textbf{Quality Control for Molecular Diagnostics} & & & \\
QCMD1 & 3.00 & 2.34 & 2.57 \\
QCMD2 & 4.00 & 3.31 & 3.70 \\
QCMD3 & 2.40 & 1.53 & 0.00 \\
QCMD4 & 2.00 & 1.41 & 0.00 \\
QCMD5 & 3.40 & 2.70 & 2.76 \\
QCMD6 & 3.00 & 2.44 & 2.17 \\
QCMD7 & 2.40 & 1.30 & 0.00 \\
QCMD8 & 3.70 & 2.88 & 3.25 \\
QCMD9 & 0.00 & 1.00 & 0.00 \\
QCMD10 & 2.70 & 2.20 & 2.14 \\
\hline
\textbf{American Society for Transplantation} & & & \\
EBV03 & 2.30 & 0.00 & 1.68 \\
EBV04 & 3.91 & 4.07 & 3.76 \\
EBV05 & 3.30 & 2.72 & 3.15 \\
EBV06 & 5.30 & 2.69 & 2.97 \\
EBV07 & 4.04 & 4.87 & 4.71 \\
EBV08 & 0.00 & 0.00 & 0.00 \\
EBV09 & 1.30 & 0.00 & 1.36 \\
EBV10 & 3.30 & 2.45 & 2.84 \\
EBV11 & 4.30 & 3.81 & 4.15 \\
EBV12 & 4.16 & 4.18 & 4.23 \\
\hline
\end{tabular}
\caption{Consensus, Laboratory-Developed Assay, and Artus Assay Values for the Quality Control for Molecular Diagnostics and American Society for Transplantation Proficiency Panels}
\end{table}
the LOQ as the lowest concentration that could be quantified with an SD of 0.15 log_{10} copies/mL or less to be 3.90 log_{10} copies/mL. The assay was linear over 3.8 log of the tested range, with the limiting factor being the initial concentration of Namalwa cells in our dilution series; however, real-time PCR assays typically have dynamic ranges of 5 to 6 log. The Artus assay has an acceptable level of reproducibility, with the CV of the high and low patient samples of 2% or less. There is good agreement between the laboratory-developed and Artus methods, with an overall bias of –0.35 log_{10} copies/mL, indicating that the laboratory-developed assay gives a lower result than the Artus assay.

One limitation of this study was the fact that the patient samples tested were refrigerated for up to 7 days before testing, which might result in sample degradation. However, data in our laboratory indicate that EBV is stable in whole blood for up to 1 week at 4°C (data not shown).

An advantage of the Artus ASR is that it provides laboratories with commercially prepared standards and reagents produced using good manufacturing practices. Given that no standard method or international standard material exists by which EBV viral load may be quantified, the accuracy of any assay is open to question, as such precision remains a key measure of assay performance. The ability for multiple laboratories to use centrally manufactured components in their assays should improve the comparability of assays between laboratories and increase the ability of laboratories to generate consensus threshold values for clinical diagnosis and intervention.

A recent study by Hayden et al\(^6\) showed a high degree of variability in viral load values between different laboratory-developed assays. This variability was seen even though many of the laboratory assays had good intralaboratory reproducibility. The interlaboratory variability was decreased when common QSSs were provided to the laboratories.\(^8\) This study underscores the need for an international standard for EBV quantification to improve the agreement in viral load values obtained from different laboratories. Moving forward, it is likely that the best intralaboratory and interlaboratory reproducibility of viral load values will be obtained when an international standard and standardized reagents are available.

References


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Supported in part by grant P30 AI050409 from the Emory University Center for AIDS Research.