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Evaluation of the new Abbott Real-Time EBV assay: fully automated quantification of EBV in whole blood by targeting *BLLF1*

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ABSTRACT

The accurate measurement of the Epstein–Barr virus (EBV) DNA level in the blood is required for managing EBV-associated diseases. A new commercial Abbott Real-Time EBV assay, which targets the *BLLF1* gene, was evaluated on 120 clinical whole blood samples and the Qnostics EBV analytical panel. The limit of detection of the assay was 48.9 IU/mL (95% confidence interval, 48.1–49.8 IU/mL). The assay was linear from 2 to 5 $\log_{10}$ IU/mL ($R^2 = 0.997$). The within-run coefficients of variation (CVs) ranged from 1.68% to 4.75% and the between-run CVs ranged from 1.73% to 12.83% for samples with high, medium, and low viral loads. EBV DNA loads measured by Abbott EBV assay strongly correlated with the results quantified by another commercial Nanogen EBV Real-Time Alert Q-PCR assay ($r = 0.879$, $P < 0.0001$). The fully automated Abbott Real-Time EBV assay targeting *BLLF1* reduced both hands-on time and turnaround time and demonstrated a reliable performance for EBV DNA quantification in whole blood.

1. Introduction

Epstein–Barr virus (EBV) belongs to the Herpesvirus family and is subclassified within the Gammaherpesvirinae subfamily. This virus favorably infects B lymphocytes by attaching to the CD21 receptor on their surface via viral envelope glycoprotein gp350/220 (Nemerow et al., 1987; Young and Rickinson, 2004). In the general population, EBV seropositivity reaches 90% in adults where the virus genome often establishes a persistent and asymptomatic infection within the host (Ruf and Wagner, 2013). Occasionally, EBV infection manifests as a self-limiting lymphoproliferative disease, i.e., infectious mononucleosis. EBV is also associated with the development of several malignancies, including posttransplant lymphoproliferative disorder (PTLD), nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma, non-Hodgkin's lymphomas (e.g., Burkitt lymphoma and T/NK cell lymphoma), and gastric carcinoma (Ruf and Wagner, 2013).

The measurement of the EBV viral load in the blood (whole blood or plasma) using DNA amplification techniques is critical for diagnosis, the initiation of preemptive therapy, and monitoring the response to the therapy, particularly in high-risk PTLD and NPC patients. Real-time PCR assay has been used as a standard for the quantification of EBV DNA loads. However, previous reports have stated that interlaboratory variation for real-time PCR results exists and that the assay performance still remains to be improved (Fryer et al., 2016; Hayden et al., 2008; Preiksaitis et al., 2009; Rychert et al., 2014). To improve the agreement of interlaboratory results, the first WHO international standards (IS) were recently established, and international units (IU) were introduced to provide the standardization of viral load values independent of the applied methods (Fryer et al., 2016).

In this study, we evaluated the analytical performance of the fully automated commercial Abbott Real-Time EBV assay (Abbott Molecular, Des Plaines, IL), which combines automated DNA extraction on the m2000sp system and real-time amplification of EBV DNA on the m2000rt PCR system. In this assay, the results were expressed in IU/mL. To our knowledge, this is the first report identifying the analytical performance of this real-time EBV assay, which targets the *BLLF1* gene, on whole blood.

2. Methods and materials

2.1. Clinical samples

We examined 120 consecutive whole-blood samples for evaluating between-run imprecision and for comparing the methods; these samples were collected from 97 patients, including 23 hematopoietic stem cell transplant recipients, 29 solid organ transplant recipients, and 15 patients with lymphoma or leukemia, and were previously analyzed

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using the Nanogen EBV Real-Time Alert Q-PCR assay (Nanogen Advanced Diagnostics, Italy) between December 2015 and January 2016. The whole blood specimens were stored at −80 °C for up to 2 months. The study was approved by the institutional review board of the Seoul National University Hospital.

2.2. Analytical standards

We used the Qnostics EBV analytical panel (catalog number: EBVAQP02, ISO standard 13,485:2012) (Qnostics, Ltd., Scotland, United Kingdom) for assessing the limit of detection (LOD), linearity, and within-run imprecision. This panel included 1 negative control and 8 positive members spanning 1.86–5.56 $\log_{10}$ IU/mL.

2.3. Real-time PCR assays

The Abbott Real-Time EBV assay was performed according to the manufacturer's instructions. This assay targets the *BLLF1* gene, which encodes the gp350/220 envelope glycoprotein. The Abbott Real-Time EBV assay includes 3 reagent kits: a reagent kit for amplifying the EBV target sequence; a control kit that includes negative, low positive, and high positive controls; and a calibrator kit that includes 2 calibrators for setting the standard curve. Briefly, 300 μL of whole blood was placed on the automatic Abbott m2000sp instrument for nucleic acid extraction. A total of 250 μL of DNA was eluted, of which 35 μL was automatically combined with 25 μL of a master mix solution. After sample preparation, the plate was immediately transferred to the Abbott m2000rt instrument for PCR amplification and detection. The results were interpreted as follows: target not detected, target detected but not quantifiable (<2.18 $\log_{10}$ IU/mL), target detected and quantifiable (2.18 $\log_{10}$ IU/mL to 8.30 $\log_{10}$ IU/mL), and target detected above the upper limit of quantification (>8.30 $\log_{10}$ IU/mL). According to the manufacturer, the conversion factor between IU/mL and copies/mL is 1.46 for whole blood.

Nanogen EBV Real-Time Alert Q-PCR assay (Nanogen) was performed according to the manufacturer's instructions. After executing DNA isolation from the whole blood using a MagNa Pure 96 DNA and Viral NA Small Volume Kit (Roche, Indianapolis, IN) on a MagNa Pure 96 instrument (Roche), the amplification was carried out by real-time PCR targeting EBV Epstein–Barr nuclear antigen-1 (*EBNA1*) gene.

2.4. Analytical evaluation

2.4.1. LOD

The LOD was estimated using the Qnostics EBV analytical EBVAQP151B sample (5.56 $\log_{10}$ IU/mL). The sample was serially diluted in EBV-negative whole blood, with expected EBV DNA loads of 3×10^3, 1×10^3, 3×10^2, 1×10^2, 3×10^1, and 1×10^1 IU/mL. Ten replicates for each concentration were analyzed. The LOD was defined as the lowest concentration of the EBV DNA, detected with 95% probability.

2.4.2. Linearity

Linearity was determined using a serially diluted Qnostics EBV analytical EBVAQP151B sample (5.56 $\log_{10}$ IU/mL) in EBV-negative whole blood with expected EBV DNA loads ranging from 2 to 5 $\log_{10}$ IU/mL. Two replicates for each concentration were tested. The mean concentration and the standard deviation were calculated.

2.4.3. Reproducibility

Within-run imprecision was examined with high, medium, and low viral loads by using serially diluted Qnostics EBV analytical EBVAQP151B sample (5.56 $\log_{10}$ IU/mL) at expected values of 3.5, 3.0, and 2.5 $\log_{10}$ IU/mL. Between-run imprecision was determined using 10-fold dilution of an EBV-positive clinical sample (972,684 copies/mL, previously quantified with Nanogen EBV Real-Time Alert Q-PCR assay) with expected values of 4.7, 3.7, and 2.7 $\log_{10}$ IU/mL. Ten replicates were tested for within-run and between-run imprecision: For the between-run imprecision, the replicate aliquots were examined in 5 consecutive days. Each sample was divided into multiple 300-μL aliquots: each aliquot was stored at −20 °C without thawing up to 5 days. For each sample, coefficient of variation (CV) was estimated.

2.4.4. Agreement

A total of 120 clinical samples were quantified with Abbott Real-Time EBV assay and Nanogen EBV Real-Time Alert Q-PCR assay for assessing the agreement between the 2 assays.

2.5. Statistical analysis

The LOD was calculated by probit analysis. The linearity was determined by plotting the observed values against the expected values based on the linear regression model. The agreement of the quantitative results between the 2 real-time PCR assays was established by the Passing–Bablok regression. The Pearson correlation coefficient (r) was used to estimate the correlation between the 2 assays. The concordance between the 2 assays in the qualitative results was determined by Cohen's kappa. Additionally, the bias between the 2 assays was estimated by the Bland–Altman plot. All tests were 2-sided, and a P value of <0.05 was considered significant. Analyses were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA), SPSS 19.0 software (SPSS 19.0, Chicago, IL), and MedCalc 17.6 software (MedCalc Software, Ostend, Belgium).

3. Results

3.1. LOD

The dilution series of the EBV analytical panel confirmed an LOD of 48.9 IU/mL [95% confidence interval (CI), 48.1–49.8 IU/mL] (Table 1). The detection rates for dilutions with expected values of 3×10^3, 1×10^3, 3×10^2, 1×10^2, 3×10^1, and 1×10^1 IU/mL were 100%, 100%, 100%, 100%, 80%, and 50%, respectively.

3.2. Linearity

The assay was linear from 5 to 2 $\log_{10}$ IU/mL ($R^2 = 0.998$; $y = 1.019x - 0.0831$). The result, representative of the linearity of the assay, is presented in Fig. 1.

3.3. Reproducibility

Reproducibility was examined by testing 10 replicates (within-run imprecision) and 2 replicates of 5 independent runs (between-run imprecision). The within-run CVs were 1.68%, 3.46%, and 4.75% for high viral load (mean, 3.72 $\log_{10}$ IU/mL), medium viral load (mean, 3.23 $\log_{10}$ IU/mL), and low viral load (mean, 2.69 $\log_{10}$ IU/mL), respectively (Table 2). The between-run CVs were 1.73%, 3.60%, and 12.83% for the high viral load (mean, 4.31 $\log_{10}$ IU/mL), medium viral load

Table 1
LOD of the Abbott Real-Time EBV assay using the whole blood sample

EBV analytical panel (IU/mL)	Abbott assay, mean (IU/mL)	Abbott assay, SD (IU/mL)	No. of positive results/no. of tested samples
3000	5306	741	10/10 (100%)
1000	1740	458	10/10 (100%)
300	514	147	10/10 (100%)
100	149	58	10/10 (100%)
30	37	19	8/10 (80%)
10	24	9	5/10 (50%)

LOD = limit of detection; EBV = Epstein–Barr virus; SD = standard deviation; No. = number; IU = international unit.

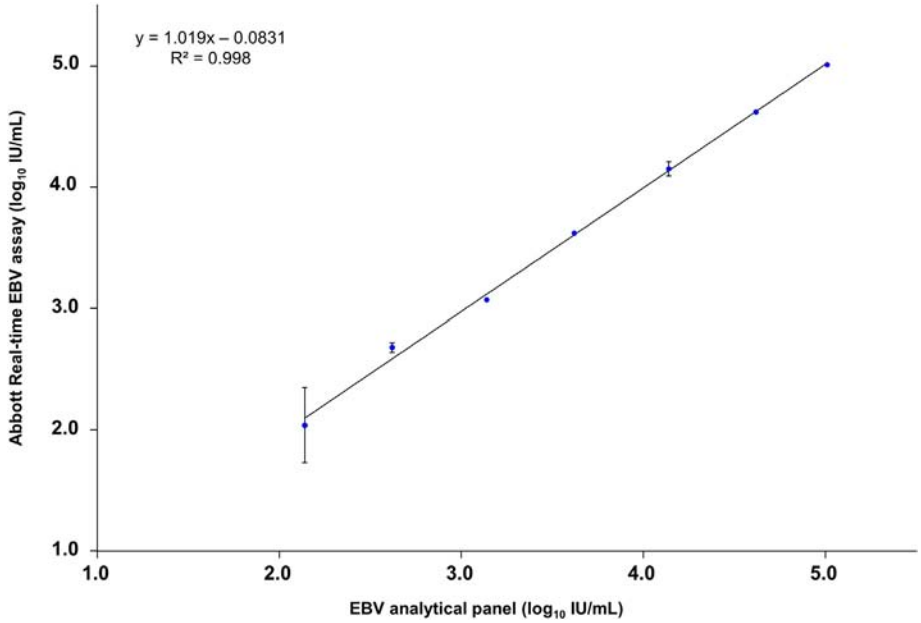

Fig. 1. Linear range of the Abbott Real-time EBV assay. The mean values for each concentration of 5, 4.5, 4, 3.5, 3, 2.5, and 2 log10 IU/mL were presented, with error bars representing the standard deviation. A linear regression line ($y = 1.019x - 0.0831$) and R^2 indicate that this assay is linear across this range.

(mean, 3.64 $\log_{10}$ IU/mL), and low viral load (mean, 2.85 $\log_{10}$ IU/mL), respectively (Table 3).

3.4. Agreement

The Abbott Real-Time EBV assay detected EBV DNA in 105 out of 119 (88.2%) clinical samples; 1 sample was excluded from data analysis because it yielded an invalid result through the Abbott Real-Time EBV assay. The EBV DNA was quantifiable (>2.18 $\log_{10}$ IU/mL) in 98 out of 119 (82.3%) samples. The Nanogen EBV Real-Time Alert Q-PCR assay was able to detect and quantify EBV DNA in 100 out of 119 (84.0%) clinical samples. These data indicate that the Abbott Real-time EBV assay was detectable for 4.2% increased samples and quantifiable for 1.7% decreased samples compared with the Nanogen EBV Real-Time Alert Q-PCR assay.

The 2 assays showed very good agreement ($\kappa = 0.825$, $P < 0.01$). EBV DNA was detected in 100 samples by both assays. EBV DNA was undetectable in the 14 samples by both assays. Five samples tested positive only in the Abbott Real-Time EBV assay, 2 of which were quantifiable (mean viral load by Abbott assay, 2.63 $\log_{10}$ copies/mL; range, 2.44–2.82 $\log_{10}$ copies/mL). For the samples that could be quantified by both assays, the EBV DNA loads were significantly correlated between the assays ($r = 0.879$, $P < 0.0001$) (Fig. 2). Linear regression analysis confirmed the absence of a constant systematic error and proportional error; the 95% CI of the intercept includes the value 0 (−0.4875 to 0.3323), and the 95% CI of the slope includes the value 1 (0.9451–1.1649). The Bland–Altman plot showed a positive bias of 0.11 $\log_{10}$ copies/mL for the Abbott Real-Time EBV assay when compared with the Nanogen EBV Real-Time Alert Q-PCR assay (Fig. 3).

4. Discussion

The quantification of the EBV DNA load is the best strategy for initiating preemptive rituximab to prevent PTLD and monitoring the response to therapy in patients with EBV-associated diseases. Thus, in clinical laboratory assays, highly sensitive and reproducible quantification is required, and a standard result reporting system (e.g., IU) is needed. In this regard, we investigated the analytical performance of the Abbott Real-Time EBV assay measured in IU/mL.

The LOD for the Abbott Real-Time EBV assay was 48.9 IU/mL, which was lower than the value provided by the manufacturer (115.2 IU/mL of whole blood). As the WHO IS were used in the evaluation performed by the manufacturer, the use of different analytical panels may be a possible reason for the slight discrepancy. We often experience that the LOD indicated in the package insert tends to be higher than the LOD results obtained in the laboratory. This assay exhibited higher sensitivity for detecting EBV DNA in whole blood compared with the previously evaluated different commercial kits (Buelow et al., 2016; Hubner et al., 2015). Since the dynamics of EBV DNA load are critical for the decision to initiate preemptive therapy, the reproducibility of the quantitative analysis should be considered. In this regard, the within-run and between-run imprecision findings of this assay showed a trend toward higher CV with lower EBV DNA loads. These findings are comparable with those of previous studies which evaluated other commercial EBV DNA quantification assays (Hubner et al., 2015; Raggam et al., 2010).

We compared the Abbott Real-Time EBV assay with the Nanogen EBV Real-Time Alert Q-PCR assay using 120 clinical whole blood samples. These 2 assays are strongly correlated with a bias of 0.11 $\log_{10}$

Table 2
Evaluation of within-run precision

Sample	Number of tests	Mean concentration ($\log_{10}$ IU/mL)	SD ($\log_{10}$ IU/mL)	CV (%)
Panel 1	10	3.72	0.06	1.68
Panel 2	10	3.23	0.11	3.46
Panel 3	10	2.69	0.13	4.75

IU = international unit; SD = standard deviation; CV = coefficient of variation.

Table 3
Evaluation of between-run precision

Sample	Number of tests	Mean concentration ($\log_{10}$ IU/mL)	SD ($\log_{10}$ IU/mL)	CV (%)
1	10	4.31	0.07	1.73
2	10	3.64	0.13	3.60
3	10	2.85	0.37	12.83

IU = international unit; SD = standard deviation; CV = coefficient of variation.

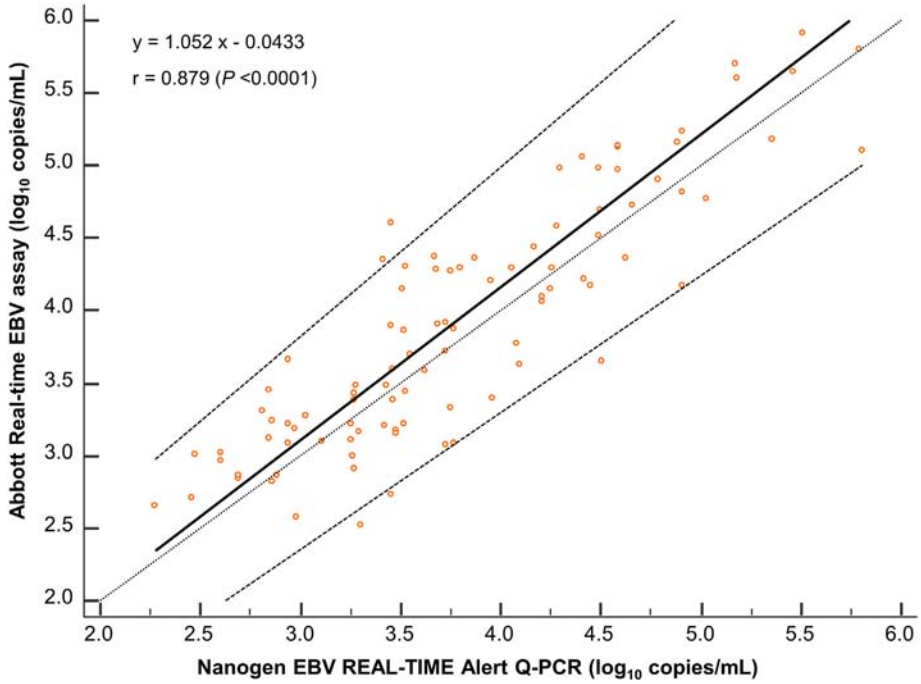

Fig. 2. Correlation between EBV viral loads determined by the Abbott assay and the Nanogen assay. The Passing–Bablok linear regression analysis showed the intercept of −0.0433 and the slope of 1.052. The solid line represents the regression line, and the dashed lines represent the 95% CI.

copies/mL, which demonstrates that the Abbott Real-Time EBV assay yields higher test results than the Nanogen EBV Real-Time Alert Q-PCR assay. In terms of detection abilities, 5 discrepant results between the 2 assays were observed, particularly in specimens with low EBV DNA loads. The reasons for the discrepancy were not evident; however, the different DNA extraction systems we used in each assay may have led to the differences in extraction efficiency and caused the difference in DNA quantification results. Although a conversion factor provided by the manufacturer of the Abbott Real-Time EBV assay was applied to compare 2 assays, the discrepant results emphasize the need to express results in IU/mL to standardize EBV DNA quantification.

Currently, there is no consensus on the optimal specimen for measuring EBV DNA load. However, the use of unfractionated whole blood provides several advantages over the use of plasma. Unfractionated whole blood contains all blood compartments and thus directly reflects the absolute EBV viral load in the patient's circulation (Stevens et al.,

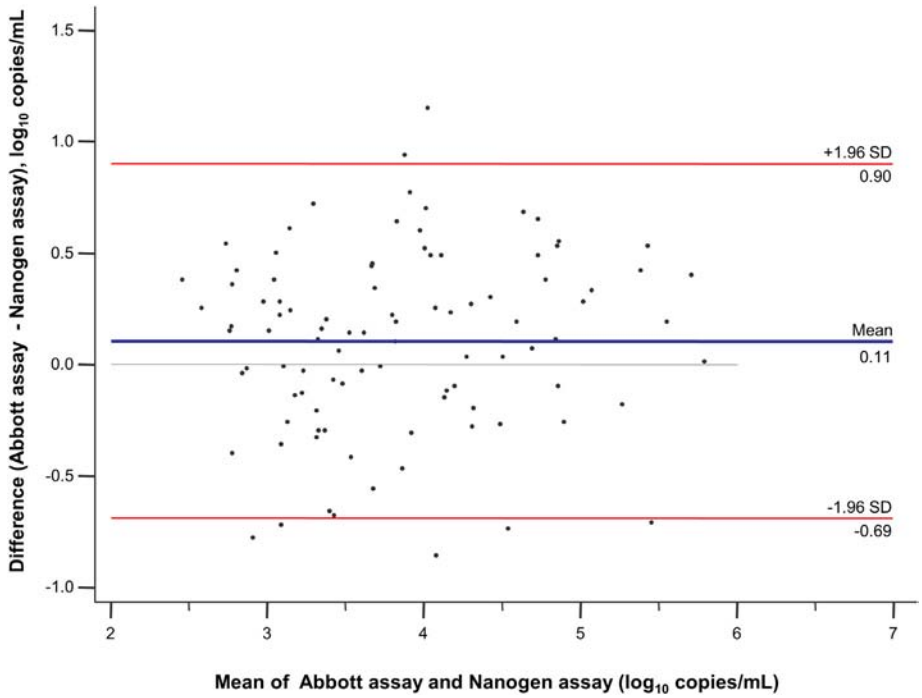

Fig. 3. Bland–Altman analysis of 96 matched EBV-positive samples in both assays. Navy line represents the mean difference (bias) of 0.11. The red lines represent the ±1.96 standard deviations (95% CI).

2001). Since most EBV-associated complications in immunosuppressed transplant patients (e.g., posttransplant lymphoproliferative disease) are cell associated, whole blood is preferred over plasma in monitoring these patients (Kimura et al., 2008). A recent study by Lazzarotto et al. analyzed the kinetics of EBV DNA load in paralleled whole blood and plasma samples and suggested the higher sensitivity of whole blood than plasma for monitoring posttransplantation EBV infection (Lazzarotto et al., 2018).

Another practical advantage of whole blood is that it avoids the additional centrifugation step required for preparing plasma, thus saving both time and labor in clinical laboratories (Gulley and Tang, 2010). The use of whole blood is also suitable for quantifying other viral targets of interest (e.g., cytomegalovirus and human herpes 6 virus) in monitoring transplant recipients (Salmona et al., 2016).

Commercial reagents for quantitative EBV DNA measurement have displayed a wide range of target genes (e.g., *EBNA-1*, *BNRF1*, *BXLF1*, and *LMP2*) (Gulley and Tang, 2010). To the best of our knowledge, few previous studies have reported analytical performance of the Real-Time EBV assay which targets the *BLLF1* gene. Another recent study by Vinuesa et al. reported that the plasma EBV DNA loads measured by the Abbott Real-Time EBV assay and by another commercially available artus EBV PCR assay (Qiagen, MD, USA) were comparable (Vinuesa et al., 2017). *BLLF1* encodes glycoprotein 350/220, which is most widely expressed in the EBV envelope. This protein binds the CR2 receptor and mediates EBV's entry into the B lymphocytes (Sitompul et al., 2012). A previous study by Hayden et al. described that the amplification of different target genes contributed to the differences in mean EBV DNA load results between commercial reagents (Hayden et al., 2012). This indicates the benefits of using a standard target gene for progress in standardizing EBV DNA measurement and yielding more informative results as therapeutic and prognostic parameters.

Owing to the retrospective design of this study, there was a limitation in using aliquoted clinical samples stored at −80 °C for up to 2 months. This is a single-center study, which would need to be repeated in other laboratories to determine the interlaboratory variation of this assay. The advantages of the Abbott Real-Time EBV assay are that it enables fully automated DNA extraction and amplification, which reduces both hands-on time and the turnaround time: The turnaround time required for 48 samples is approximately 7 h, of which only 1 h is related to hands-on activity. Approximately turnaround time of 2 h, with a hands-on time of 2 h, can be saved by using the Abbott Real-Time EBV assay. This assay also provides the DNA quantification results in IU/mL, which could lead to the standardization of EBV DNA quantification.

In summary, this study indicates that the Abbott Real-Time EBV assay exhibited a reliable analytical performance for EBV DNA quantification in whole blood. Our results highlight the advantages of this fully automated assay that uses international unit standards, making it suitable and favorable for use in clinical laboratories.

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