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Measuring hepatitis B pgRNA stability using an updated automated HBV pgRNA assay with increased sensitivity

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Abstract

Background: HBV pregenomic RNA (pgRNA) is a circulating biomarker for covalently closed circular DNA activity in HBV-infected individuals and has been studied for treatment efficacy, disease staging, and off-therapy outcomes; however, data on the stability are scarce. Increasing HBV pgRNA assay sensitivity may improve its predictive value and provide additional insights at low viral levels.

Methods: Modifications to a fully automated first (v1) generation HBV pgRNA assay improved sensitivity up to 15-fold over the previous assay. Flexible sample input volumes yielded lower limits of quantitation of 10 and 22 copies/mL for 0.6 and 0.2 mL assays, respectively. Results are standardized to secondary standards that are traceable to the WHO HBV DNA standard, and internal and external controls are included.

Results: Comparison between v1 and modified v2 assays showed increased sensitivity from 152 copies/mL with v1 to 10 (0.6 mL) and 22 (0.2 mL) copies/mL with v2, respectively. Quantitated v2 results were indistinguishable from v1, indicating that comparisons can be made to previous studies. Single timepoint treatment-naive blood donors or longitudinal draws from patients with chronic hepatitis B on AB-729, an investigational siRNA therapy, showed improved detection and quantifiable pgRNA with v2 compared with v1. Stability testing demonstrated excellent HBV pgRNA plasma stability after 3 freeze-thaw cycles, for at least 7 days at 25–37 °C and at least 30 days at 4°C, with $\leq$0.25 Log U/mL decrease.

Conclusion: HBV pgRNA v2 assays with increased sensitivity and flexible input volumes demonstrated increased detection and quantitation of low viral titer samples. Highly sensitive HBV pgRNA assays may be useful in refining predictive treatment outcomes based on this marker. HBV pgRNA was stable under multiple conditions, which increases the reliability of this marker.

Abbreviations: cccDNA, covalently closed circular DNA; CHB, chronic HBV infection; NA, nucleos(t)ide analog; pgRNA, pregenomic RNA.

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INTRODUCTION

HBV is a blood-borne pathogen of major global health importance, with an estimated 296 million people chronically infected, 1.5 million new infections, and more than 800,000 related fatalities in 2019 alone.[1] The replication lifecycle of HBV, in particular, the production of high levels of antigenemia inducing a state of antiviral immune tolerance and the establishment of covalently closed circular DNA (cccDNA) replication reservoirs in the hepatocyte, results in chronic HBV (CHB) infection in some individuals, particularly children. This chronic infection can persist for many years, eventually leading to fibrosis, cirrhosis, HCC, and/or liver failure. HBV undergoes a complicated lifecycle whereby virions containing the relaxed circular DNA HBV genome infect susceptible hepatocytes through binding of the Sodium Taurocholate Co-transporting Polypeptide through the pre-S1 domain of the HBsAg.[2] Within the infected hepatocyte, the relaxed circular DNA is transformed into the highly stable cccDNA that serves as the platform from which all of the HBV mRNA and pregenomic RNA (pgRNA) transcripts are produced, the latter of which can be packaged into nascent virions together with the HBV polymerase and secreted from the infected cell.[3,4] Given its global importance, a number of serologic and molecular diagnostic assays have been developed to identify and track the course of HBV infections, including evidence of past exposure. These include serologic assays for HBsAg and HBeAg antigens, present in the blood of infected individuals, and anti-HBc, which measures antibodies raised against the HBV core antigen, indicating previous HBV exposure. Molecular assays directly measuring the presence of HBV DNA in the blood are also routinely used in the screening of infections and monitoring patients on therapy.

The goal of HBV therapy is to achieve a functional cure, currently defined as unquantifiable HBsAg with or without anti-HBs seroconversion after stopping all therapies. Currently, the only approved therapies for the treatment of CHB infection are nucleos(t)ide analog (NA) compounds and pegylated interferon, which only results in a functional cure in 3%–5% of patients. NA blocks the ability of the HBV reverse transcriptase to transcribe the pgRNA into relaxed circular DNA. These potent compounds lead to the rapid reduction of HBV DNA to undetectable levels in the plasma of most patients and reduce the progression of liver damage associated with the disease.[5–7] Importantly, NA does not directly act on the cccDNA present in infected hepatocytes, which can be long-lived, and HBV mRNA, pgRNA, and viral proteins, including HBsAg, continue to be produced in most patients on therapy. Upon cessation of NA therapy, this lasting reservoir of active cccDNA can lead to a reactivation of the virus and the necessity to restart therapy. Thus, individuals with CHB must remain on NA therapy for extended periods of time, sometimes lifelong. Therefore, new finite HBV therapies capable of producing higher rates of functional cure are needed. As these experimental therapies enter the development pipeline, it has become clear that new HBV biomarkers will be needed to assess their efficacy and predict clinical outcomes. Given that HBsAg can be produced from the transcriptional activity of cccDNA as well as integrated HBV DNA, this biomarker does not directly reflect cccDNA activity in patients, and reports of HBsAg seroreversion in functionally cured patients after HBsAg loss have been described.[8] Additional HBV biomarkers which directly indicate the continued activity of cccDNA in hepatocytes are needed. One such experimental biomarker which has shown promise in identifying ongoing cccDNA activity is hepatitis B pgRNA, which is present in the peripheral circulating blood of HBV-infected individuals. HBV pgRNA has been demonstrated to circulate at ~1–2 logs below HBV DNA in untreated CHB patients.[9–11] Importantly, NA therapy does not directly affect the production of pgRNA-containing virions, and its continued presence can act as a surrogate marker for ongoing cccDNA activity in treated patients.[12] HBV pgRNA has been assessed in a number of studies looking at its clinical utility in disease staging,[13–16] therapy efficacy,[17,18] and its ability to predict off-treatment outcomes.[10,19–21]

We previously described the development of a fully automated quantitative assay for the detection of HBV pgRNA with a lower limit of quantitation of 1.65 Log U/mL (~152 copies/mL)(HBV RNA v1).[22] As more studies assess the potential utility of HBV pgRNA, it may be necessary to enhance the sensitivity and precision to be able to further explore the potential biological relevance of HBV pgRNA in novel therapies and off-treatment outcomes, particularly at low viral titer levels. Furthermore, the stability of HBV pgRNA should be confirmed in additional studies and at longer time intervals to further validate the marker as a viable analyte that can be compared across studies.[23] Here we report on a modified HBV pgRNA assay, hereafter HBV RNA v2, with increased sensitivity and precision. We show improved analytical sensitivity and quantifiable RNA in clinical samples with low HBV RNA levels compared to HBV RNA v1 results. Finally, we report HBV RNA stability under various storage conditions and freeze-thaw cycles.

METHODS

Samples

Human plasma or serum samples were obtained from the following sources: CHB patients for which treatment was documented (Boca Biolistics, LLC, Pompano Beach, FL), Cameroon blood donor plasma samples positive for HBsAg and HBV DNA, and archived

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longitudinal samples from the study AB-729-001 (Arbutus Biopharma) where patients received multiple doses of an investigational HBV-targeting siRNA (60 mg every 8 wk) and NA therapy. The study protocol and informed consent documents were approved by the applicable health authority and ethics committee/institutional review board for each country and site, and all the subjects gave written informed consent before any screening procedures in accordance with Good Clinical Practice and the Declaration of Helsinki. Institutions obtained ethics approval for the research protocols, and all patients provided written informed consent. Samples originating from the Cameroon study were approved by the Cameroon National Ethical Review Board, the Faculty of Medicine and Biomedical Science IRB, and the Ministry of Health of Cameroon.

Detection of HBV pgRNA

HBV pgRNA was quantitated using the HBV RNA v1 assay as described.[22] Briefly, HBV RNA is isolated from 0.2 mL of a sample using RNA-selective extraction chemistry (Abbott *m*Sample Preparation System, Abbott Molecular), followed by multiplex real-time quantitative PCR. Amplified targets in the HBV X and core regions are detected on the *m*2000 system (Abbott Molecular). Standardization of assay results is achieved by calibration using DNA-extracted HBV DNA secondary standards (Abbott Molecular) traceable to the World Health Organization's HBV DNA standard such that 1 U of reported HBV RNA is equivalent to 1 IU of HBV DNA. An armored RNA internal control (Abbott Molecular) is used to validate extraction and RNA amplification in each sample. External controls are also extracted with each run to confirm the expected performance of HBV RNA target amplification.

Modifications to assay formulation, sample extraction volume (0.2 or 0.6 mL), and PCR amplification cycles were assessed and resulted in an HBV RNA v2 assay with increased performance (earlier Cts, reduced Ct variance among replicates, and more consistent detection of low copy number samples) compared with HBV RNA v1. Primers and probes for targets in the X and core were not modified from the HBV RNA v1 assay as described.[22] HBV RNA v2 sensitivity and linearity were evaluated on replicates of serial dilutions of a clinical sample with high initial HBV pgRNA (8.00 Log U/mL) at 6 (n = 3), 5 (n = 3), 4 (n = 3), 3 (n = 20), 2.3 (n = 20), 2 (n = 20), 1.7 (n = 20), 1.4 (n = 20), 1.1 (n = 20), 0.8 (n = 20), and 0.49 (n = 20) Log U/mL HBV pgRNA concentrations. Results from HBV RNA v2 (0.2 mL) and HBV RNA v2 (0.6 mL) performance testing were compared with HBV RNA v1.

Evaluating HBV RNA v2 sensitivity

Single timepoint HBsAg and HBV DNA positive samples from untreated Cameroonian blood donors were tested on HBV RNA v1 and 2 (0.2 and 0.6 mL) assays. HBV RNA results were compared to HBV DNA viral load (Abbott RealTime 0.2 mL DNA, Abbott Molecular) testing results.

Assay performance with low HBV RNA viral copy samples was assessed on archived longitudinal samples from clinical trial AB-729-001. Briefly, patients were HBeAg negative, on NA therapy, and receiving an investigational HBV-targeting siRNA therapy. HBV pgRNA measurement was performed with both HBV RNA v1 and HBV RNA v2 0.6 mL assays for comparison.

HBV pgRNA stability

An HBV pgRNA stability study was conducted to assess the impact of storage conditions and freeze-thaw cycles on HBV RNA recovery. A clinical sample with a high initial pgRNA concentration (8.00 Log U/mL) was diluted to 2 expected concentrations of 5.5 and 3 Log U/mL and aliquoted into replicates. Aliquoted samples were stored

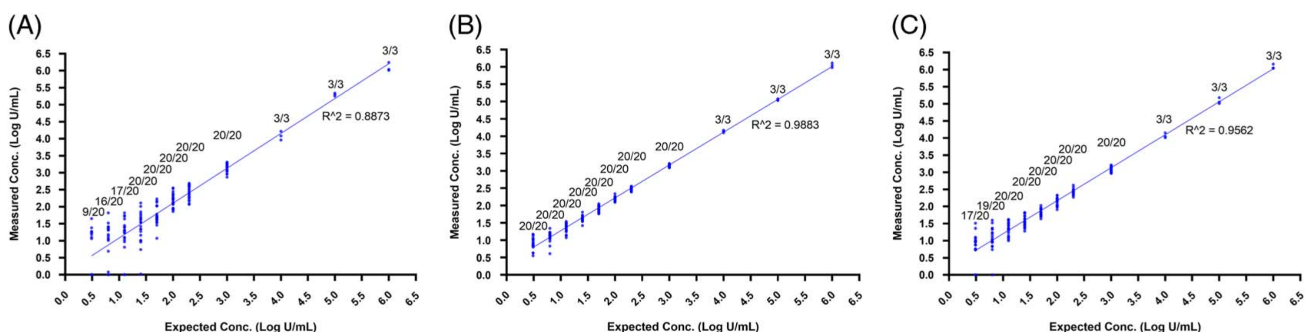

FIGURE 1 Sensitivity comparison between HBV RNA v1 and HBV RNA v2 (0.6 mL and 0.2 mL). Quantitated HBV RNA results at expected HBV RNA concentrations from serial dilutions for HBV RNA v1 (A), HBV RNA v2 (0.6 mL) (B), and HBV RNA v2 (0.2 mL) (C). Numbers above each expected concentration indicate the number of detected samples divided by the number of tested samples. R^2 values were calculated across all tested concentrations in GraphPad Prism 8.0.2.

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at −20, 4, 25, and 37 °C for 8 hours, 1, 2, 3, 5, 7, 14, 21, and 30 days before freezing at −80 °C until testing was performed. Samples stored at 25 and 37 °C were collected out to day-7. Baseline aliquoted samples were immediately frozen at −80 °C until testing. Samples undergoing freeze-thaw testing were initially frozen at −80 °C before being thawed 1, 2, or 3 times and kept at 25 °C for 30 minutes (after completely thawing) before refreezing at −80 °C. Stability samples were tested in replicates of n = 4.

Statistical analysis

All statistical analysis and graphing were performed in GraphPad Prism 8.0.2. Where indicated, Mann–Whitney *U*, descriptive statistics (median, mean, and SD), and linearity were calculated in GraphPad Prism. A *p* value < 0.05 was considered significant.

RESULTS

HBV pgRNA assay modifications increase sensitivity and precision

A fully automated dual-target assay for the quantitation of HBV pgRNA (HBV RNA v1) and a lower limit of quantitation of 1.65 Log U/mL (152 copies/mL) was reported.[22] We wondered whether modifications to the assay formulation, sample input volume, and/or amplification conditions could increase assay performance (sensitivity and precision). After optimization studies were performed, an HBV RNA v2 assay with either 0.6 mL or 0.2 mL sample input volumes was completed. The limit of detection (95% detection) was extrapolated from Probit analysis to be 0.24 Log U/mL (6 copies/mL) and 0.80 Log U/mL (22 copies/mL) for the 0.6 mL and 0.2 mL input volumes, respectively. Limits of quantitation (LOQ, 20% coefficient of variation) were determined to be 0.48 Log U/mL (10 copies/mL) for the 0.6 mL input volume and 0.8 Log U/mL (22 copies/mL) for the 0.2 mL input volume (Supplemental Table 1, http://links.lww.com/HC9/A211).

A comparison of sensitivity between HBV RNA v1 and v2 (0.6 mL and 0.2 mL) assays was performed on replicates of serial dilutions of a high HBV RNA sample (Figure 1A–C). Results from 20 tested replicates indicated increasing numbers of undetected samples starting at 1.1 Log U/mL (43 copies/mL) with the v1 assay and ending in 9/20 (45%) detection at 0.49 Log U/mL (11 copies/mL). In contrast, 20/20 (100%) of samples at 0.49 Log U/mL (11 copies/mL) concentrations were detected with v2 0.6 mL, and 19/20 (95%) were detected at 0.8 Log U/mL (22 copies/mL) with v2 0.2 mL. Linearity across the tested dilution range was computed to be $R^2 = 0.8873$, 0.9883, and 0.9562 for HBV RNA v1, v2 0.6 mL, and v2 0.2 mL, respectively. Mean quantitated results of serial dilutions ranging from 6.00 Log U/mL to 1.70 Log U/mL were compared between HBV RNA v1 and HBV RNA v2 (0.6 mL and 0.2 mL) assays to demonstrate that results generated with each assay are comparable to each other (Figure 2). Differences between mean quantitated values with each assay were within the SD of HBV RNA v1 results for each tested concentration except at 5 Log U/mL, which was slightly higher in v1 (Supplemental Table 2, http://links.lww.com/HC9/A211). Coefficients of variation (%CV) ranged from 0.5% to 4.5% and 1.1%–5.8% for 0.6 mL and 0.2 mL assays, respectively, between the range of 1.7–6.0 Log U/mL (Supplemental Table 2, http://links.lww.com/HC9/A211).

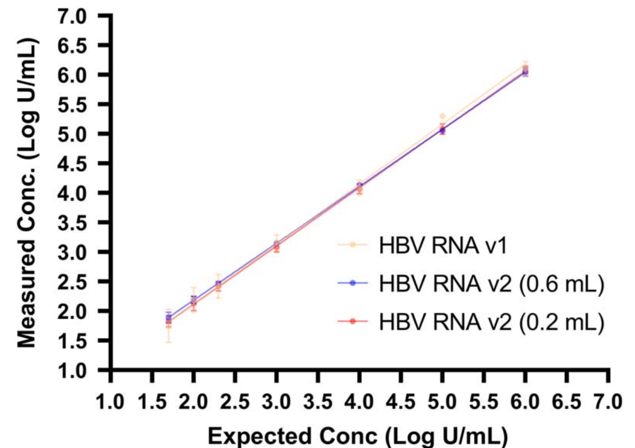

FIGURE 2 Quantitated HBV RNA is comparable between HBV RNA v1 and HBV RNA v2 assays. Mean Log U/mL HBV RNA values at expected concentrations ranging from 1.7 to 6.0 Log U/mL were compared between HBV RNA v1 (green), HBV RNA v2 0.6 mL (blue), and HBV RNA v2 0.2 mL (red). Mean values were obtained from n = 20 replicates of concentrations 1.7–3.0 Log U/mL and from n = 3 replicates of concentrations 4.0–6.0 Log U/mL. Vertical error bars indicate SDs.

Increased detection and quantitation of HBV pgRNA in untreated infections with HBV RNA v2

To assess the performance of the assays on HBV clinical samples, a panel of untreated single timepoint blood donors (n = 40) collected from Cameroon tested with HBV RNA v1, v2 0.6 mL, and v2 0.2 mL. HBV RNA (Log U/mL) was compared with HBV DNA (Log IU/mL) results showing the correlation with R^2 values of 0.8601, 0.9049, and 0.8520 for HBV RNA v1, v2 0.6 mL, and v2 0.2 mL, respectively (Figure 3). As expected, HBV DNA was quantitated 1–2 Log IU/mL higher than HBV RNA in most samples. Importantly, improvements in the number of samples with detectable HBV RNA were observed with v2 0.6 mL (35/40, 87.5%) and v2

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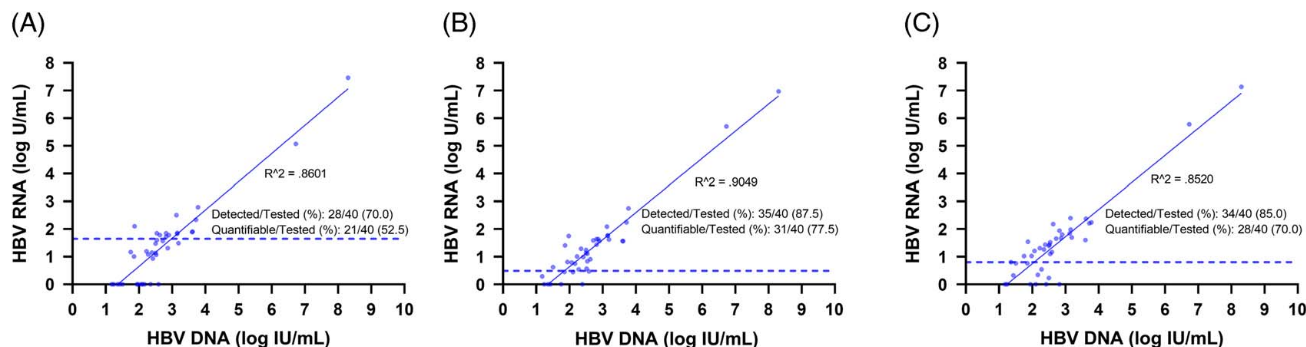

FIGURE 3 Comparison of HBV RNA and HBV DNA levels in untreated blood donors. HBV RNA v1 (A), HBV RNA v2 0.6 mL (B), and HBV RNA v2 0.2 mL (C) Log U/mL results (y-axis) were compared to HBV DNA (x-axis) results from n = 40 untreated Cameroonian blood donors. Linear regression and corresponding R^2 values were calculated in GraphPad Prism 8.0.2. Dotted horizontal lines indicate the limit of quantitation for each HBV RNA assay version. The number of detected/tested (%) and the number of quantifiable/tested (%) samples for each assay are indicated on their respective graphs.

0.2 mL (34/40, 85%), compared with v1 (28/40, 70.0%). In addition, the number of samples with quantifiable levels of HBV RNA was higher with v2 0.6 mL (31/40, 77.5%) and v2 0.2 mL (28/40, 70.0%) compared with v1 (21/40, 52.5%).

HBV RNA v2 shows increased sensitivity and precision in patients undergoing therapy with low levels of HBV RNA

To assess the performance of the assays on HBeAg (−) CHB patients undergoing therapy, longitudinal samples collected from 3 patients on a combination NA + HBV-targeting siRNA investigational therapy were tested with HBV RNA v1 and v2 0.6 mL. Patients were dosed with 60 mg of HBV-targeting siRNA AB-729 at every 8-week intervals. In contrast to NA, which do not directly affect HBV RNA, siRNAs target HBV RNA for degradation by the RNA interference pathway and have been shown to reduce plasma HBV RNA.[24] In this study, patients displayed low baseline levels of pgRNA, which were further reduced upon AB-729 dosing. More samples were detected with the v2 (37/39, 94.9%) than the v1 (17/39, 43.6%) assay providing a more granular assessment of HBV RNA decline kinetics. (Figure 4A–C). Importantly, only 1/39 (2.6%) samples had quantifiable levels of HBV RNA with v1 compared with 31/39 (79.5%) when v2 0.6 mL was used.

HBV pgRNA is stable under various storage conditions and freeze-thaw cycles

The ability to use HBV RNA as a robust biomarker will depend on the stability characteristics that it displays under various storage and handling conditions. To assess the relative stability of HBV RNA, a clinical sample with a high initial HBV RNA titer was diluted to 2 concentrations (~5.5 Log U/mL and 3 Log U/mL), aliquoted, and stored under various temperature conditions (−20, 4, 25, and 37 °C) for up to 7 to 30 days and 1, 2, or 3 freeze-thaw cycles. Results from replicates (n = 4) at each condition showed less than a 0.5 Log U/mL drop from baseline levels after 7 days at 25 and 37 °C, up to 30 days at −20 and 4 °C, and after 3 freeze-thaw cycles demonstrating robust stability of HBV RNA under each tested condition (Figure 5). The absolute difference between baseline and subsequent timepoints was ≤ 0.25 Log U/mL in all cases and not considered biologically meaningful (Supplemental Table 3, http://links.lww.com/HC9/A211).

DISCUSSION

Here we have reported on a version 2 HBV pgRNA assay with increased sensitivity and precision and the ability to use either 0.6 mL or 0.2 mL sample input volumes. Importantly, results generated with both v2 assays are comparable to those generated with the previously described v1[22] assay allowing for study comparisons across assay versions. This is achieved through the use of secondary standards traceable to the WHO HBV DNA international standard used to calibrate each assay in the absence of a recognized HBV RNA standard. Further, the automated process from sample extraction to RT-qPCR reduces sample-to-sample and run-to-run variation, and the use of both internal and external validity controls ensures that assay performance is nominal for each run. Another advantage is the flexible input volume which is useful for studies that require the highest sensitivity (10 copies/mL using v2 0.6 mL) versus studies where sample volume might be limiting (22 copies/mL using v2 0.2 mL).

We have also shown that increasing the sensitivity of the HBV pgRNA assay resulted not only in an increase in the number of detectable samples but also in

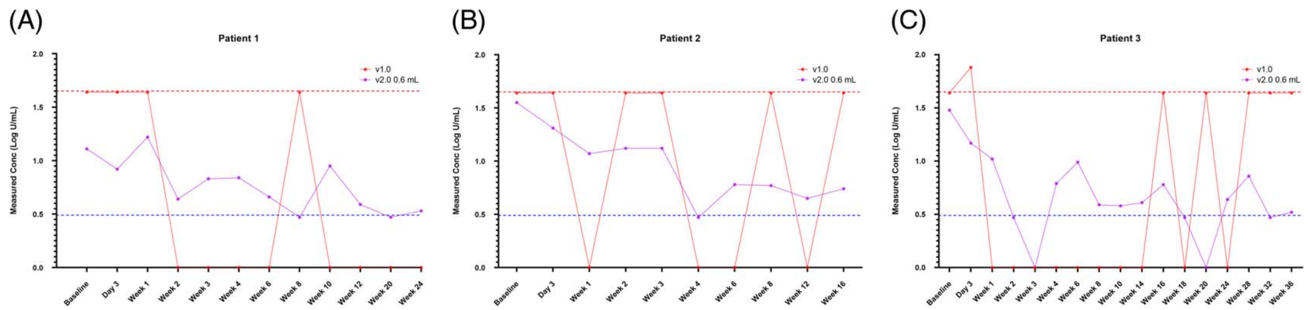

FIGURE 4 Comparison of longitudinal HBV RNA levels in patients undergoing NA and investigational HBV-targeting siRNA therapy. Longitudinal HBV RNA v1 (red) and HBV RNA v2 0.6 mL (blue) Log U/mL results were determined for 3 individual patients (A–C) on a combination NA/siRNA therapy. Study day/week timepoints are indicated on the x-axis. Horizontal red and blue lines indicate the limit of quantitation for HBV RNA v1 and HBV RNA v2 0.6 mL, respectively. Samples with detected HBV RNA results below the defined assay limits of quantitation were adjusted to just below the LOQ; 1.64 Log U/mL and 0.47 Log U/mL for HBV RNA v1 and HBV RNA v2 0.6 mL, respectively. Samples with no detectable HBV RNA were assigned a value of 0 Log U/mL.

the number of quantifiable results in both untreated and treated individuals. Furthermore, the improved precision, specifically with low HBV RNA-containing samples allows for greater visibility into small longitudinal changes within individuals and could provide additional insights into the activity of investigational HBV therapies.[25]

As more and more studies investigate the potential for HBV RNA use in staging disease,[13,15] tracking therapy efficacy,[12,18] and predicting off-therapy outcomes[10,19,20] it is becoming increasingly important that well-characterized and standardized automated assays are utilized when addressing these questions.[26] Publications reporting HBV pgRNA measurements should clearly describe sensitivity, precision, limits of quantitation, and how the assay validation and calibration were achieved. Furthermore, an HBV RNA international standard would be very useful in allowing to further standardize study results and draw conclusions across different studies.[27] These issues will be particularly important as assays measuring HBV pgRNA become increasingly sensitive and/or if certain levels of HBV pgRNA are found to be predictive of disease outcomes or treatment outcomes, including new therapeutic modalities.

To further validate HBV RNA as a potential biomarker, it is important to understand how stability is affected under various storage conditions. A recent report assessed HBV RNA stability at 4 and 25 °C for up to 2 days and 4 freeze-thaw cycles and observed minimal changes in HBV RNA recovery.[23] Here, we have demonstrated that HBV RNA is similarly stable at −20 and 4 °C for at least 30 days and stable at 25 and 37 °C for at least 7 days and after 3 freeze-thaw cycles. Together, these results show that HBV RNA is a stable biomarker even under elevated temperatures and that short-term sample storage conditions are unlikely to be a concern for study results.

In conclusion, HBV RNA has shown promise in identifying ongoing cccDNA activity in patients on NA therapy, in enabling examination of new mechanisms of action of investigational therapies, and in predicting patients who may experience a viral rebound after therapy cessation.[19,20] The ability to detect HBV RNA with increased sensitivity and precision may provide additional

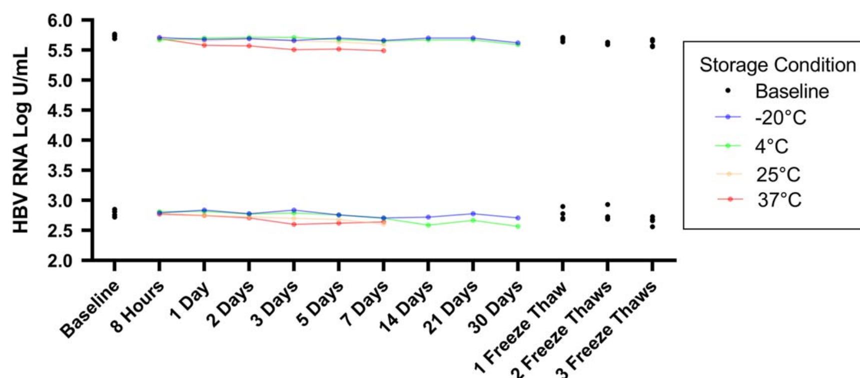

FIGURE 5 HBV RNA storage and freeze-thaw stability. A high titer HBV RNA sample was diluted to two expected concentrations (5.5 and 3 Log U/mL), aliquoted, and stored at the indicated conditions for the indicated amounts of time before freezing at −80 °C before testing. Replicates (n = 4) of each condition were tested with HBV RNA v2 0.6 mL, and mean values and connecting lines are reported for −20 °C (blue), 4 °C (yellow), 25 °C (green), and 37 °C (red). Individual HBV RNA replicate values (n = 4) are shown for baseline and 1–3 freeze-thaw cycles.

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benefit in establishing the value of this biomarker in assessing disease outcomes and treatment outcomes.

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CONFLICT OF INTEREST

Mark Anderson, Michael Stec, and Gavin Cloherty are employees and shareholders of Abbott Laboratories. Emily P. Thi and Gaston Picchio are employees and shareholders of Arbutus Biopharma. The remaining authors have no conflicts to report.

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