Development of a Highly Sensitive Multiplex Platform Assay to Monitor Low Levels of HBV DNA and pgRNA in Samples from Patients with Chronic Hepatitis B

Qi Huang, Ran Yan, Dawei Cai, Ariel Tang, Xiang Xu and Richard Colonno

Assembly Biosciences, Inc., South San Francisco, CA, United States

Introduction

- HBV cure rates remain low despite prolonged Nucleos(t)ide (NrtI) therapy, likely due to persistent residual viral replication on therapy and an inability to eliminate cccDNA pools
- Recent studies indicate that serum HBV DNA levels (a surrogate for virus replication) combined with serum pregenomic RNA (pgRNA) levels (a surrogate for cccDNA levels), as an emerging predictor of sustained post-treatment response[1,2]
- Current assays, such as the COBAS (AmpliPre/Cobas TaqMan HBV test v2), quantitate HBV DNA levels down to 20 IU/mL, but are not able to monitor loss of residual virus in HBV patients on NrtI therapy[3,4]
- There are no approved commercial assays available to detect plasma HBV pgRNA levels[5]
- New sensitive assays are clearly needed to assess treatment efficacy and guide therapy discontinuation decisions
- We have developed a panel of highly sensitive nucleic acid assays designed to monitor levels of HBV DNA, pgRNA and total nucleic acids (composite DNA+pgRNA) in clinical samples

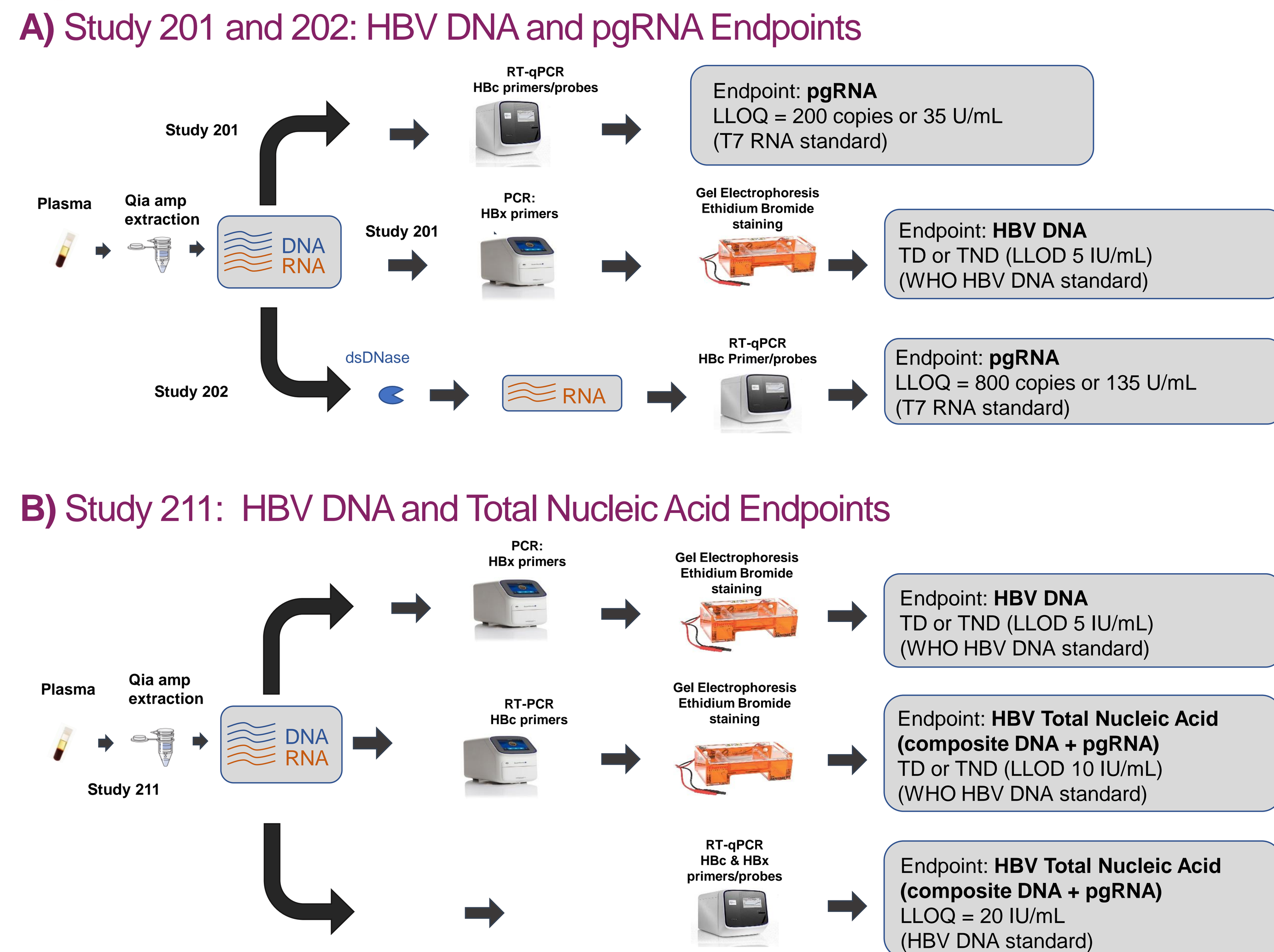
Materials and Methods

- **Samples:** Patient samples from ongoing ABI-H0731 (Vebicorvir; VBR) Phase 2a studies, including virologically-suppressed (n=73) and treatment naïve (n=25) CHB patients enrolled in ongoing ABI-H0731 Phase 2 studies were tested (EASL 2020 Abstracts 4256 and 4603)
- **Nucleic Acid Extraction:** Viral nucleic acids were purified from 500 µL of patient plasma using QIAamp MinElute Virus Vacuum kits
- **Detection of HBV DNA or Total Nucleic Acids (TNA):** Highly sensitive PCR (HBV DNA) and RT-PCR (composite DNA+pgRNA) assays were developed and calibrated against the AcroMetrix HBV standard. Both PCR products (284 bp HBx region) and RT-PCR (577 bp HBc region) were sequenced to confirm that they corresponded to the intended HBV sequence of each patient at baseline or screening
- **Quantification of HBV DNA, pgRNA and Total Nucleic Acid (TNA):** For (RT)-qPCR assays, the detection of amplified DNA was performed using two HBV target-specific (HBc and HBx) primers, and HBc target results were reported, in an approach similar to recent published methods[5]
- **Standard & Controls:** The following standards were used, where indicated for specific assays: T7-transcribed HBV (Gt-B) RNA; Linearized HBV (Gt-B) plasmid; AcrometrixTM HBV control (calibrated to 1st WHO international HBV standard)[6]

Figure 1. Study Design

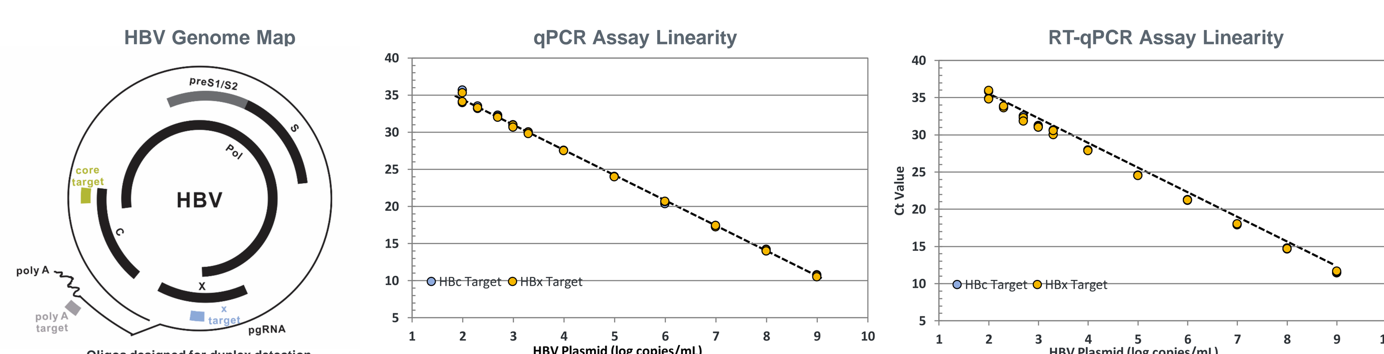
Figure 2. Flow chart

A) Study 201 and 202: HBV DNA and pgRNA Endpoints



B) Study 211: HBV DNA and Total Nucleic Acid Endpoints

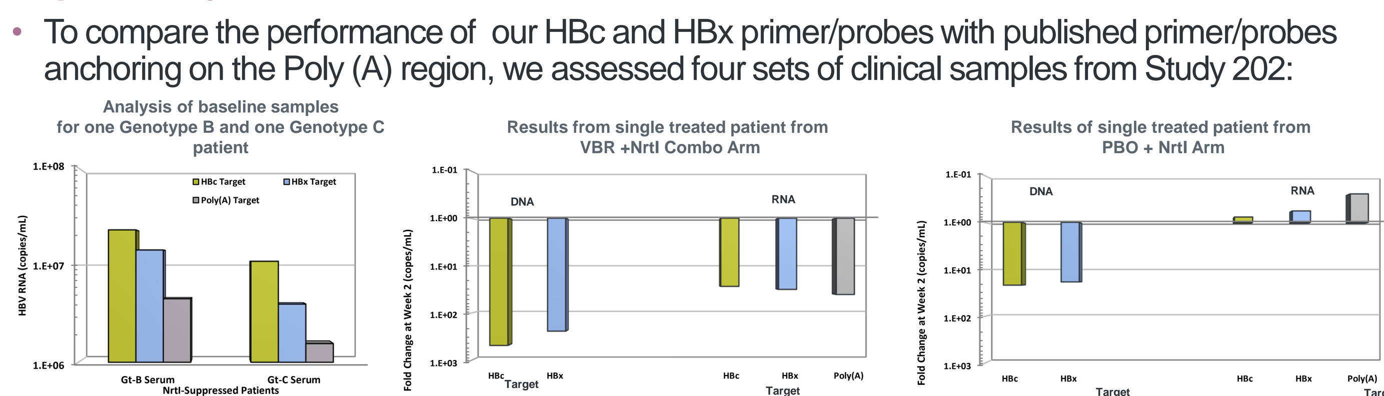
Figure 3. Primer design and dynamic range



- For quantification of HBV DNA and pgRNA, primers and probes were designed to pan-genotypic, conserved regions within the 3' end of the Core gene (125 bp) and the 5' end of the X gene (99 bp), similar to other published methods[5]
- Primers with poly (A) anchoring were also designed for comparative study[3]
- Linearity evaluated in duplicate using 10-fold dilution of an HBV Gt-B plasmid standard (n=2, 2–9 log copies/mL)
- Both the HBV DNA (qPCR) and pgRNA (RT-qPCR) assays exhibited good range and linearity (R^2> 0.99 in both assays)

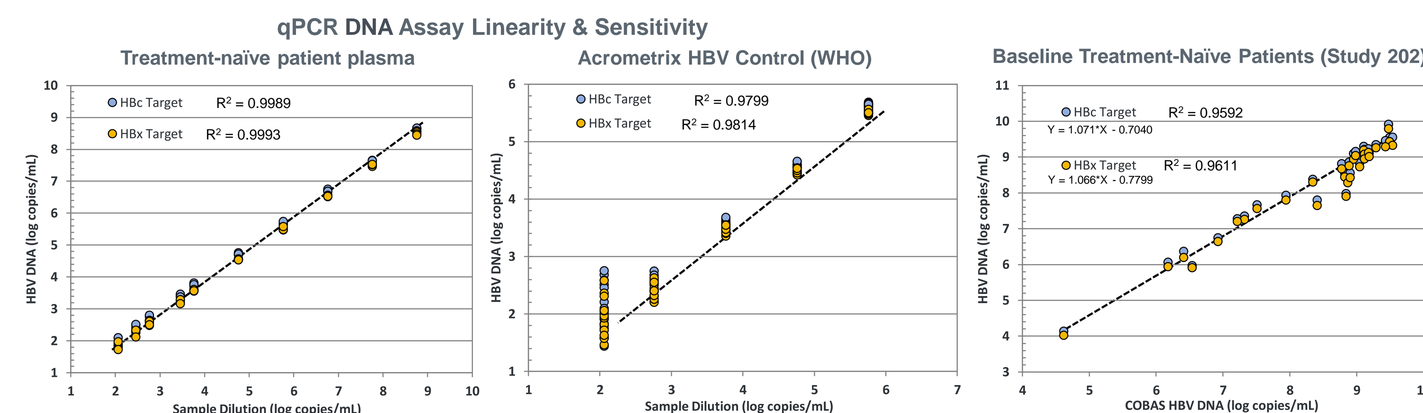
Figure 4. pgRNA primer comparator studies

- To compare the performance of our HBc and HBx primer/probes with published primer/probes anchoring on the Poly (A) region, we assessed four sets of clinical samples from Study 202:



- pgRNA measured in 2 baseline treatment-naive patients (Study 202) using 3 sets of primer/targets
- For pgRNA quantification, targeting HBc is more sensitive than targeting HBx
- The RACE method using poly (A) anchor[3] showed even less sensitivity, consistent with recent results showing pgRNA has shorter heterogeneous lengths with 3' truncations
- HBV DNA and pgRNA measured at baseline and week 2 in a patient receiving VBR+NrtI
  - A significant decline in HBV DNA was measured at 2 weeks with by both primer/targets
  - A significant decline in pgRNA was measured at 2 weeks with all three primer/targets at 2 weeks, as VBR blocks pgRNA encapsidation
- HBV DNA and pgRNA measured at baseline and week 2 in a patient receiving placebo+NrtI alone
  - A significant decline in HBV DNA was measured at 2 weeks with by both primer/targets
  - pgRNA levels increased slightly, consistent with the mechanism of action of NrtIs which allow encapsidation but block reverse transcription thus stabilizing pgRNA

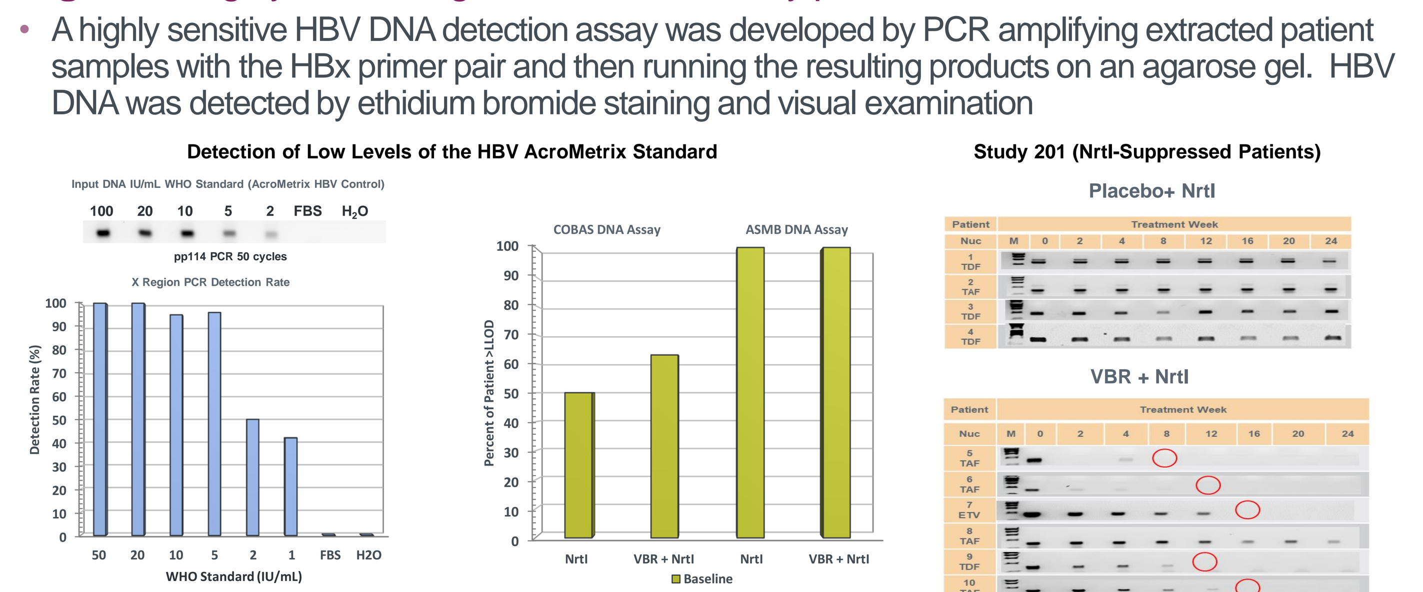
Figure 5. qPCR DNA assay performance



- The linear range and sensitivity of the ASMB HBV DNA assay was validated by testing:
  - A serially diluted clinical HBV plasma sample (left panel, n=4 repeats)
  - An AcroMetrix HBV control (1-5 log IU/mL) (middle panel, n=10–20 repeats)
- A LLOQ of 20 IU/mL was determined by serial dilution of two HBV samples: serial dilution of CHB Plasma tittered by COBAS, with HBV Standard and full range of linearized GT-B plasmid (0-7 logs) in each experiment; 1 IU = 5.82 copies DNA
- The ASMB assay had a dynamic range and sensitivity equivalent to the COBAS assay
- 31 Baseline samples from treatment-naïve patients with HBV GTs A, B, C & E were tested by both COBAS and the ASMB qPCR DNA assay
- Performance was similar for the COBAS assay and both the HBc and HBx primer/probe sets for the ASMB assay across the range of HBV DNAs present in these samples.

Figure 6. Highly sensitive gel-based DNA assay performance

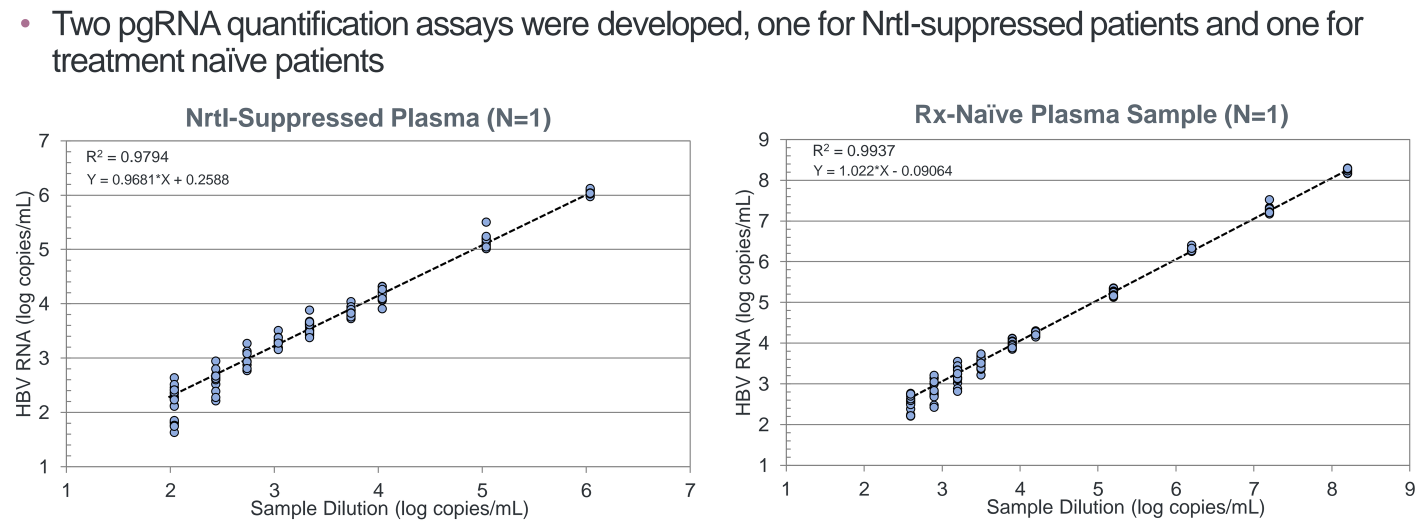
- A highly sensitive HBV DNA detection assay was developed by PCR amplifying extracted patient samples with the HBx primer pair and then running the resulting products on an agarose gel. HBV DNA was detected by ethidium bromide staining and visual examination



- The AcroMetrix DNA control was reproducibly detected at 5 IU/mL (96% n=47 tests) which was set as the LLOD
- Results from testing baseline samples from Study 201: the Assembly DNA assay was frequently able to detect HBV when the COBAS DNA assay was not
- Examining longitudinal samples from patients in Study 201 indicated:
  - Patients receiving placebo+NrtI had detectable HBV DNA at all time points
  - Patients receiving VBR+NrtI more often became undetectable for HBV DNA (red circles highlight time point at which samples went below 5 IU/mL)

Figure 7. pgRNA assay performance

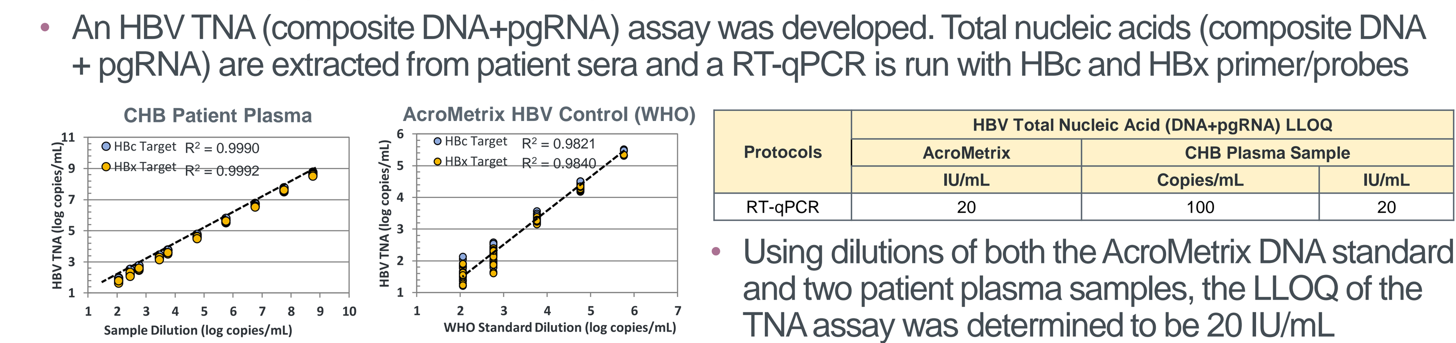
- Two pgRNA quantification assays were developed, one for NrtI-suppressed patients and one for treatment naïve patients



- For **Study 201** (NrtI-suppressed patients with **low HBV DNA** (COBAS <20 IU/mL) and **high pgRNA**):
  - A RT-qPCR was run using HBc primers/probe.
  - The LLOQ is 200 copies/mL or 35 U/mL (1 U = 5.82 copies/mL RNA)
  - T7 RNA used as Standard
  - The linear range is 2 to 6 logs copies/mL
- For **Study 202** (Rx naïve patients with **high HBV DNA** and **high pgRNA**:
  - A RT-qPCR was run using HBc primers/probe.
  - The LLOQ is 800 copies/mL or 135 U/mL (1 U = 5.82 copies/mL RNA)
  - T7 RNA used as Standard
  - The linear range is 2.6 to 8.2 logs copies/mL

Figure 8. (RT)-qPCR TNA assay performance

- An HBV TNA (composite DNA+pgRNA) assay was developed. Total nucleic acids (composite DNA + pgRNA) are extracted from patient sera and a RT-qPCR is run with HBc and HBx primer/probes

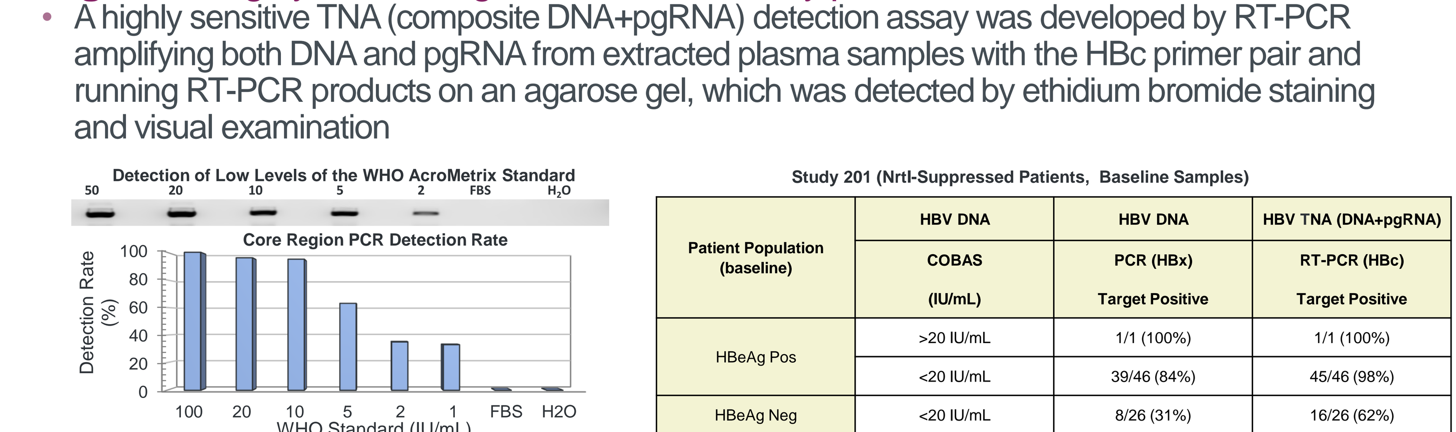


Protocols	HBV Total Nucleic Acid (DNA+pgRNA) LLOQ		
	AcroMetrix	CHB Plasma Sample	
	IU/mL	Copies/mL	IU/mL
RT-qPCR	20	100	20

- Using dilutions of both the AcroMetrix DNA standard and two patient plasma samples, the LLOQ of the TNA assay was determined to be 20 IU/mL

Figure 9. Highly sensitive gel-based TNA assay performance

- A highly sensitive TNA (composite DNA+pgRNA) detection assay was developed by RT-PCR amplifying both DNA and pgRNA from extracted plasma samples with the HBc primer pair and running RT-PCR products on an agarose gel, which was detected by ethidium bromide staining and visual examination



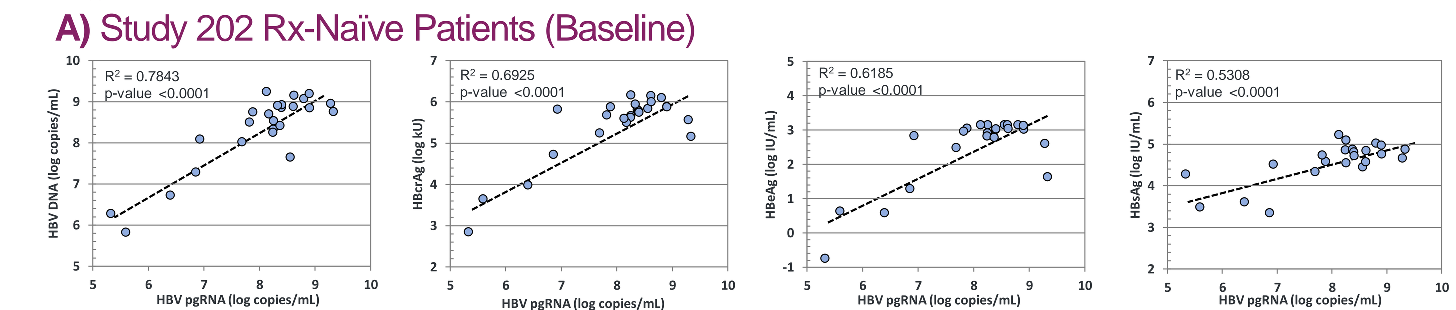
Study 201 (NrtI-Suppressed Patients, Baseline Samples)

Patient Population (baseline)	HBV DNA COBAS (IU/mL)	HBV DNA PCR (HBx) Target Positive	HBV TNA (DNA+pgRNA) RT-PCR (HBc) Target Positive
HBeAg Pos	>20 IU/mL	1/1 (100%)	1/1 (100%)
	<20 IU/mL	39/46 (84%)	45/46 (98%)
HBeAg Neg	<20 IU/mL	8/26 (31%)	16/26 (62%)

- The AcroMetrix DNA control was reproducibly detected at 10 IU/mL (95%, N=108 tests) which was set as the LLOD
- The majority of NrtI-suppressed HBeAg Pos patients were both HBV DNA and TNA positive using Assembly's assays despite being COBAS HBV DNA <20 IU/mL
- A higher percentage of NrtI-suppressed HBeAg Neg patients were TNA positive than DNA Positive, suggesting that pgRNA contributed to the TNA signal in these patients

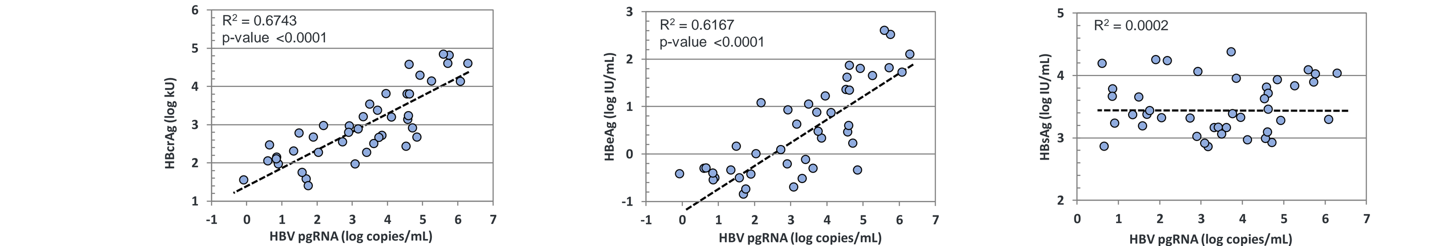
Figure 10. Baseline Biomarker Correlations

A) Study 202 Rx-Naïve Patients (Baseline)



- 25 Rx-naïve patients were analyzed for HBV DNA, pgRNA, HBsAg, HBeAg and HBcrAg levels at baseline
  - HBV pgRNA was positively correlated with other HBV biomarkers are shown above
  - Factors that may impact the correlation are sequence variability, epitope differences (HBsAg escape variants), and preCore promotor or stop codon mutations (HBeAg)

B) Study 201 NrtI-Suppressed HBeAg Pos Patients (Baseline)



- 47 NrtI-Suppressed patients with detectable pgRNA were analyzed for HBcrAg, HBeAg and HBsAg at baseline
- pgRNA was quantifiable (> 35 U/mL) in 38/47 in HBeAg positive patients; pgRNA was positively correlated with HBeAg and HBcrAg levels, but did not correlate well with HBsAg levels likely due to HBV integrants being a significant source of HBsAg in these patients

Conclusions

- Newly developed HBV DNA, pgRNA and total Nucleic Acid (composite DNA+pgRNA) assays provide greater sensitivity than currently available commercial assays
  - In NrtI treated samples from study 201, the majority of patients with HBV DNA <20 IU/mL by COBAS were found to be positive for HBV DNA or HBV Total Nucleic Acids
  - pgRNA was positively correlated with all other HBV biomarkers in naïve CHB patients; however, it did not correlate well with HBsAg in NrtI-suppressed HBeAg positive patients, likely due to HBV integrants being a significant source of HBsAg in these patients
- The multiplex HBV Nucleic Acid assay platform allows simultaneous quantification and detection of HBV DNA, pgRNA and total HBV nucleic acid may be useful in guiding treatment discontinuation decisions

References

1) Fan R, Hou J et al. Association Between Negative Results From Tests for HBV DNA and RNA and Durability of Response After Discontinuation of Nucles(t)ide Analogue Therapy. Clinical Gastroenterology and Hepatology; 2) Carey I, Gersch J, Wang B, et al. Pre-genomic HBV RNA and HBcrAg predict outcomes in HBeAg negative chronic hepatitis B patients suppressed on nucleos(t)ide analogue therapy [published online ahead of print, 2019 Nov 7]. Hepatology. 2019;10.1002/hep.31026; 3) van Bömmel F, etc Serum hepatitis B virus RNA levels as an early predictor of hepatitis B envelope antigen seroconversion during treatment with polymerase inhibitors. Hepatology. 2015;61:66–76; 4) Marcellin P, Gane EJ, Flisiak R, Manns MP, Kaita K, Gaggar A, et al. Evidence for ongoing low-level viremia in patients with chronic hepatitis B receiving long-term nucleos(t)ide analog therapy. HEPATOLOGY 2014;60:1093A; 5) Butler EK, etc . Hepatitis B Virus Serum DNA and RNA Levels in Nucleos(t)ide Analog-Treated or Untreated Patients During Chronic and Acute Infection. Hepatology. 2018; 68: 2106-2117; 6) Saldanha J, Gerlich W., Lelie N., Dawson P., Heermann K., Heath A. and The WHO Collaborative Study Group. An international collaborative study to establish a World Health Organization international standard for hepatitis B virus DNA nucleic acid amplification techniques. Vox Sanguinis 2001 80, 63-71; 7) Yuen MF et al. Safety, pharmacokinetics, and antiviral effects of ABI-H0731, a hepatitis B virus core inhibitor: a randomised, placebo-controlled phase 1 trial. Lancet Gastroenterol Hepatol. 2019 Nov 8. pii: S2468-1253(19)30346-2; 8) Ma, X. et al. Interim Safety and Efficacy Results of the ABI-H0731 Phase 2a Program Exploring the Combination of ABI-H0731 with Nuc Therapy in Treatment-Naive and Treatment-Suppressed Chronic Hepatitis B Patients. 2019 EASL.

assemblybio

Presented at the EASL Digital International Liver Conference, August 27–29 2020