Analytical and clinical performance of the high sensitive Simoa qHBsAg assay

Célia Galien, *Camila Rocaboy, Françoise Forge, Catherine Pretis, Véronique Lugaz and* Mylène Lesénéchal,
bioMérieux S.A., Marcy l'Etoile, France

Background & Objectives

Hepatitis B Virus (HBV) infection is still a serious global health problem and HBV infection is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. The qualitative measurement of Hepatitis B surface Antigen (HBsAg) is routinely used for the diagnosis of HBV infection and the screening of blood from donors, whereas the quantitative measurement of HBsAg monitors the progress of chronic hepatitis B, and its rapid decline may be a predictor of the efficacy of the antiviral therapy. However, despite a high sensitivity of the commercial HBsAg assays (0.05 IU/ml using the 2nd WHO standard) there is a continuous need to develop more sensitive assays capable of reducing the window period, detecting occult HBV carriage and monitoring patients receiving innovative treatment concepts that should offer an increased rate of functional cure.

Simoa™ (Single Molecule Array) technology provides the ability to measure protein analytes with unprecedented sensitivity, down to the femtomolar range. Quanterix's HD-1 system offers full automation of Simoa digital immunoassay technology for life science research applications. In this study, a high sensitive quantitative immunoassay for HBsAg was developed as a prototype and analytical as well as clinical usefulness was assessed on the RUO (Research Use Only) system.

Principle of Simoa Technology

In a Simoa assay (Figure 1), analyte protein molecules are captured onto antibody-coated paramagnetic beads, the captured proteins are labeled with an enzyme, and individual beads are isolated and sealed in arrays of femtoliter wells in the presence of a fluorogenic enzyme substrate.

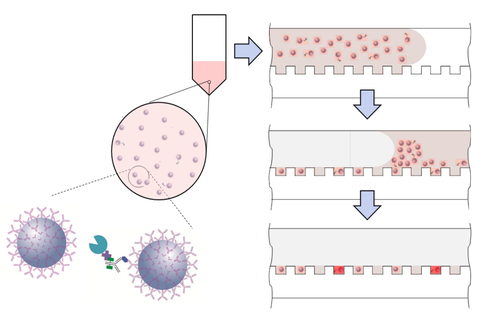

Figure 1. Basic principle of Simoa digital immunoassay technology.

Confining individual labeled immunocomplexes and substrate in fL-sized wells allows to overcome the sensitivity limitations stemming from diffusion and dilution in conventional (analog) immunoassays, and can provide a sensitivity enhancement of up to 1000-fold over traditional ELISA.

High-resolution fluorescence imaging of Simoa beaded arrays is used to determine both the fraction of beads associated with at least one enzyme and the fluorescence intensity from each well. The Simoa unit of measurement is the average number of enzymes per bead (AEB), calculated using Poisson's distribution law at ultra-low analyte concentrations (digital readout mode) or the average fluorescence intensity at higher concentrations (analog readout mode).

The Simoa HD-1 System

The Simoa HD-1 floor-standing analyzer (Figure 2) is a fully-automated instrument (samples in, results out) that integrates microbead-based ELISA robotics with a Simoa imaging module. The system consists of five main functional areas: (1) input bays for addition of disposables (tips, assay cuvettes, array discs), reagents, and samples; (2) a system bay for onboard storage of liquid resources and waste handling; (3) the user control interface (computer and touch-screen monitor); (4) a module for performing immunological reactions (incubation and wash steps); and (5) the Simoa load-seal-image (LSI) readout module for the processing of Simoa arrays. Pipetting operations are provided by two x-y-z pipettors: a disposable-tip pipettor for sample handling and transfer of bead-substrate solution to the array disc, and a fixed-tip pipettor for reagent pipetting functions.

The instrument is designed to minimize operator intervention. Results are automatically calculated and can be displayed in real time upon completion of each sample's assay. The touch-screen provides the user with access to all instrument functionalities, including run setup, system status overview and result reports. The system accommodates 5 mL, 7 mL, and 10 mL primary tubes as well as 1 mL pediatric sample cups.

Figure 2. The Simoa HD-1 immunoanalyzer (Research Use Only version).

Materials and methods

Prototype qHBsAg assay : Simoa qHBsAg prototype assay is a 1-step digital enzyme immunoassay based on sandwich principle. The total assay duration is 49 minutes. The sample volume needed is 150 µl. The prototype was designed to detect all genotypes/serotypes as well as mutants.

Calibration : The prototype has been standardized against the NIBSC standard (code number: 12/226; WHO Third International Standard for HBsAg, subtype ayw1/adw2, genotype B4; IU/mL). Since none assay is yet standardized against this new standard, 3rd WHO calibrators were titered using Elecsys HBsAg II quant II assay. 8 calibrators ranging from 0 to 2.84 IU/ml were prepared in HBV negative human sera (anti HBs negative). Calibration was performed once a week.

Specimens : negative blood donor samples were obtained from the EFS (Etablissement Français du Sang). Specimens included sera collected on tubes containing separating gel, Li-heparin, K2-EDTA- and sodium citrate plasma. Human seroconversion panels for HBV were purchased from Boston Biomedica Inc. Precision and linearity samples were diluted in HBV negative human sera.

Analytical sensitivity was assessed against the 2nd NIBSC standard using low calibrators in digital range (0 –0.00128 IU/mL). LOD was calculated as the mean from 10 replicates + 2.5 SD (RUO LOD).

Precision study was performed according to CLSI EP5-A2 guidelines : 2 runs per day in 4 replicates each for 3 days on one instrument (n = 24).

Linearity measuring range was determined based on a <10% deviation from linearity, using one sample serially diluted in HBV negative human sera in one run and one instrument (triplicate, 5 dilution factors).

All data sets were inspected for the presence of outlier results using Grubbs test.

Results

Master curve on the whole range (digital and analog) is presented in Figure 3a. Master curve in the digital range is presented in Figure 3b. Each calibrator was tested in triplicate. The calibration curve is plot using a 4PL fit model.

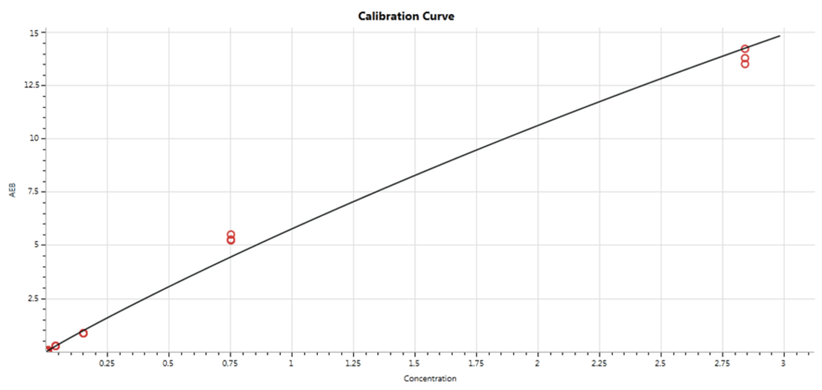

Figure 3a. Master curve on the whole range

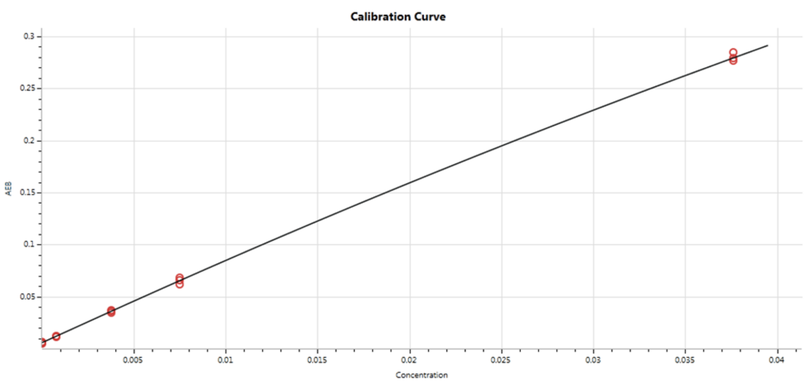

Figure 3b. Master curve on the digital range

Between-runs and days precision levels (CV) of 2.5%-11.7% are obtained for human serum specimens over the range 0.0021-0.7486 IU/mL (Figure 4).

Sample	Mean IU/mL	Repeatability CV (%)	Between runs precision CV(%)	Total precision (inter runs/days) CV (%)
Positive 1 (digital)	0.0021	5.1%	5.6%	5.9%
Positive 2 (digital)	0.0385	4.3%	7.8%	7.8%
Positive 3 (analog)	0.1226	1.8%	2.2%	2.5%
Positive 4 (analog)	0.7486	3.7%	8.1%	11.7%

Figure 4. Between-runs and day precision for 4 human serum specimens measured on one instrument during 3 days (2 runs per day, 4 replicates on each run), totalizing 24 data each sample. Assay calibration was performed on the first run. No outlier has been detected.

Limit of detection and specificity.

The LOD of the Simoa qHBsAg assay is **0.00036 IU/mL (0.36 mIU/mL) using the 2nd NIBSC standard**, which makes the assay at least 100 fold more sensitive than the Elecsys HBsAg II quant II assay (0.05 IU/mL).

To evaluate the specificity of the assay, a total of 411 negative blood donor samples were tested, among them fresh sera and frozen plasma. The spreading of the samples showed a narrow distribution in AEB (Figure 5).

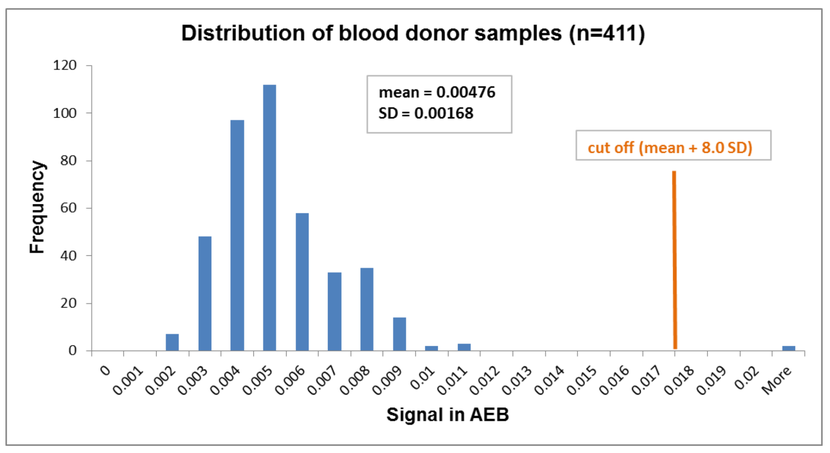

Figure 5. Distribution of blood donor samples .

In order to get an robust specificity, the cut off was defined as the mean concentration of the negative samples + 8 fold their standard deviation.

The obtained cut off corresponds 0.00154 IU/mL when using the 3rd NIBSC standard for calibration.

At this cut off, 3 initially and 2 repeatedly reactive samples were identified, respectively. Specificity in the initial assay and after retesting were **99.27 %** [97.88-99.85%] and **99.51 %** [98.25-99.94%], respectively.

Note : Using the 2nd NIBSC standard for calibration, this cut off corresponds to 0.00095 IU/ml (0.95 mIU/mL)

Linearity. Results from linearity study are shown in Figure 6. The linear measuring range of at least 3 logs was demonstrated

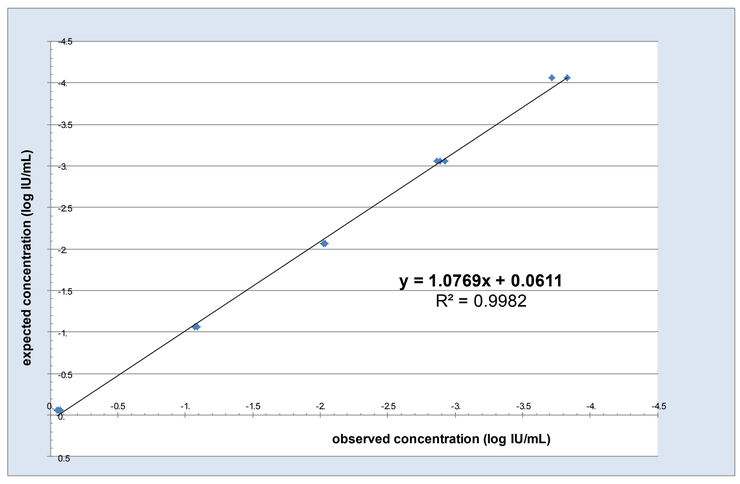

Figure 6. Linearity

Sensitivity for seroconversions. To illustrate the ability of the Simoa qHBsAg assay to detect ultra low analyte concentrations, seroconversion panels were tested. The results of the Simoa qHBsAg assay were compared with those of « state of the art » assays (Architect HBsAg QT assay, Vidas HBsAg Ultra assay) and HBV DNA PCR. The results showed that the Simoa HD-1 system has the potential to provide NAT-level sensitivity for the early detection of HBsAg, as shown in Figure 7 a/b/c.

BBI PHM 909 subtype ad	day since 1st bleed	Architect HBsAg QT assay	BBI PCR LOD 100 cp/ml	Simoa qHBsAg assay IU/mL - interpretation
PHM909-1	0	**neg**	**pos**	**0.032 / pos**
PHM909-2	4	**neg**	**pos**	**0.058 / pos**
PHM909-3	7	**neg**	**pos**	**0.132 / pos**
PHM909-4	**9**	**pos**	**pos**	**0.540 / pos**
PHM909-5	14	pos	**pos**	**1.334 / pos**
PHM909-6	18	pos	**pos**	**2.539 / pos**
PHM909-7	21	pos	**pos**	**saturation / pos**

Figure 7a. Seroconversion panel PHM 909- Simoa qHBsAg detects the Day 0 bleed vs Day 9 for Architect

PHM 911 subtype ad	day since 1st bleed	Vidas HBsAg ultra	BBI PCR LOD 100 cp/ml	Simoa qHBsAg assay IU/mL - interpretation
PHM911-13	51	neg	neg	0.0007 / neg
PHM911-14	56	neg	**pos**	0.0009 / neg
PHM911-15	58	neg	neg	0.0012 / neg
PHM911-16	63	neg	**pos**	**0.0039 / pos**
PHM911-17	65	neg	**pos**	**0.0070 / pos**
PHM911-18	70	neg	**pos**	**0.0348 / pos**
PHM911-19	72	neg	**pos**	**0.0548 / pos**
PHM911-20	**77**	**pos**	**pos**	**0.259 / pos**
PHM911-21	79	pos	**pos**	**0.532 / pos**
PHM911-22	84	pos	**pos**	**0.794 / pos**
PHM911-23	86	pos	**pos**	**1.314 / pos**
PHM911-24	91	pos	**pos**	**2.763 / pos**
PHM911-25	93	pos	**pos**	**saturation / pos**

Figure 7b. Seroconversion panel PHM 911- Simoa qHBsAg detects Day 63 bleed vs Day 77 for Vidas.

Panel	Number of days to detection from the 1st bleed		
	Architect or Vidas ¤ HBsAg	Simoa qHBsAg assay	HBV PCR
PHM909	9	**0**	**0**
PHM911	77¤	**63**	**63***
PHM922	16	**0**	**0**
PHM923	15	**0**	**0**

Figure 7c. Demonstration of the Simoa qHBsAg assay capablity to detect earlier HBsAg than other assays. * reproducibly positive HBV PCR

Conclusions & Outlook

This study demonstrates superior analytical and clinical performance of the Simoa qHBsAg prototype, with a very low limit of detection and a high specificity in agreement with clinical criterion for evaluating unknown populations. Using commercially available HBsAg seroconversion panels, Simoa qHBsAg prototype significantly shortens the window period with detection results being similar to PCR.

All together, this study shows that the Simoa qHBsAg prototype is a useful tool for an early detection screening of HBV infection and to better understand the case of occult HBV carriers as well as in the monitoring of patients receiving new antiviral therapies expected to eradicate HBV.