# Anti-HBs Assay Feasibility Report

**Theranos Inc.**

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# 1. ASSAY INFORMATION[ TC "ASSAY INFORMATION" \F C \L "2" ]

## 1.1 Assay Specifications

Hepatitis B Virus (HBV) causes the infectious illness Hepatitis. Hepatitis B may eventually cause cirrhosis and hepatocellular carcinoma. HBV is classified under the family of DNA virus *Hepadnaviridase*. The virus particle (virion) or Dane Particle is surrounded by an outer lipid protein envelope and composed of an icosahedral nucleocapsid core. It consists of a circular DNA molecule and uses a reverse transcriptase mechanism replicate. It was estimated that approximately one-third of the world population has been infected at one point in their lives. The infection of HBV can be preventable by vaccination

Anti-HBs is an antibody to the surface antigen (HBsAg) of the hepatitis B virus. It can neutralize HBV and usually perseveres for lifetime and protects against reinfection. Therefore the level of anti-HBs, the presence of hepatitis B immunoglobulin in the blood; helps to be an indicator to assess recovery from HBV infection and to assess the immune response triggered as the result of having received vaccination against the hepatitis B. Antibody concentrations of $\geq$10 mIU/mL indicate immunity to HBV.

Theranos Anti-HBs assay is designed to detect anti-HBs from plasma or serum from individual patient specimens. The assay has a reportable range from 0 to 1000 mIU/mL. Surface antigen (HBsAg) is used as capture and Alkaine phosphatase labeled-HBsAg conjugates were used as detectors. The assay format is shown in Figure 1.

## 1.2 Reference Assays [ TC "Reference Assays and Standards" \f C \l "3" ]

The following commercial ELISA kits have been used as predicate methods:

- Bio-Rad Monalisa anti-HBs EIA (Cat #25220)
- Siemens Immulite 2000 anti-HBs (Cat# L2KAB2)

## 1.3 Materials and Methods

Anti-HBs assay format is designed as a sandwich ELISA. The capture surface consists of two biotin-labeled recombinant HBs antigens coated on an avidin surface. The sample (plasma or serum) is diluted and then mixed with the detection reagents and incubated on the capture surface for 5 minutes. Then the surface is washed and the alkaline phosphatase substrate is incubated on the capture surface for 5 minutes. The resulting chemiluminescence is read in Relative Light Units (RLU) on the Theranos system.

**Figure 1: Anti-HBs assay principle**

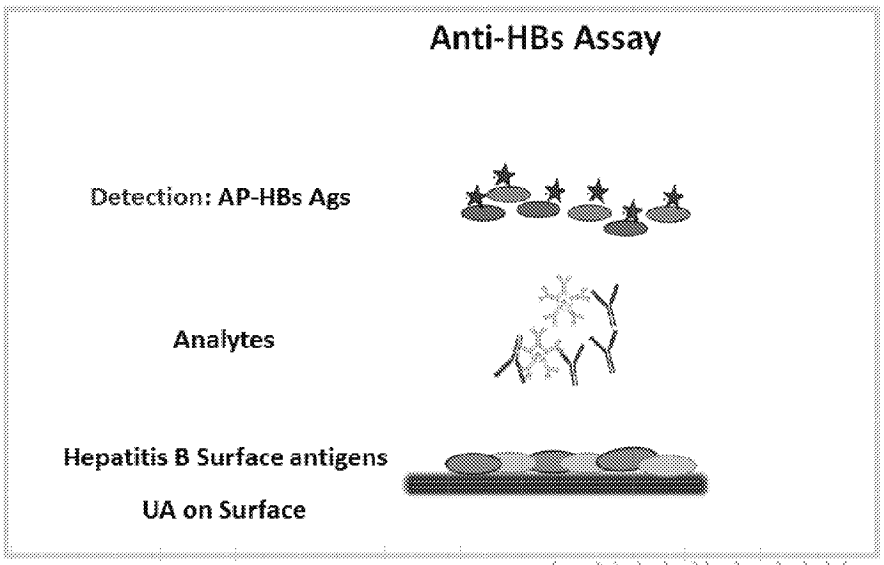



**Table 1: Materials**

| Name | Supplier | Catalog # |
|---|---|---|
| Human recombinant Hepatitis B surface adw protein (Cag1, Capture antigen 1) | Cell Sciences | CSI15712C |
| Recombinant HBsAg (ayw) (Cag2, Capture antigen 2) | Genway | GWB-A34A7B |
| WHO International Standard | NIBSC | 07/164 |
| Recombinant HBsAg (ad) (Detection conjugate 1, Dag1) | Acris | BIN142 |
| Recombinant HBsAg (ay) Detection conjugate 2, Dag2) | Acris | BIN143 |
| Alkaline Phosphatase Labeling Kit (SH) | Dojindo | LK13 |
| Biotin Labeling Kit (SH) | Dojindo | LK10 |
| Phospho Glo Substrate | KPL | 55-60-04 |
| Blocking Buffer (3% BSA in TBS, 0.05% Sodium Azide) | Sigma (BSA, Fraction V, 99% Pure) | A3059-500G |
| Carbonate-bicarbonate buffer | Sigma | C3041 |
| Stabilzyme AP stabilizer | Surmodics | SA01-1000 |

**[ TC "Reference Assays and Standards" \f C \l "3" ]**

## 2. ASSAY DEVELOPMENT [ TC "Assay Optimization" \f C \l "2" ]

### 2.1 Capture Surface Screening (MTP)

To determine the best capture surface for the Anti-HBs assay, 5 HBs Surface antigens from different vendors were selected to screen on a microtiter plate (MTP). The screening was performed with a coating of Ultravidin at a concentration of 20 ug/mL in carbonate-bicarbonate

onto a 384-well plate. Biotinylated HBs Surface antigens were added at a concentration of 5 ug/ml in blocking buffer. WHO Standard at three levels, 1000, 500, 0 mIU/mL was added as the analyte. AP-conjugated HBs Surface antigens at 100 ng/ml in blocking buffer were used as detector. The alkaline phosphatase substrate was used to develop chemiluminescent reactions. The resulting RLUs of each surface were compared (Table 2). Capture antigen 1 and 2 were highly responsive to detection 4 and detection 5. Therefore, capture antigen 1 and 2 were combined and detection 4 and 5 were combined for further evaluations (Table 3).

**Table 2: Capture antibodies screening (MTP)**

| | | Detection-AP | | | | |
|---|---|---|---|---|---|---|
| | | Dag 1 | Dag 2 | Dag 3 | Dag 4 | Dag 5 |
| **Capture Antigen** | Anti-HBsAg [mIU/mL] | Modulation | Modulation | Modulation | Modulation | Modulation |
| Cell Sciences HBsAg adw | 1000 | 20.3 | 3.1 | 1.5 | 42.5 | 62.2 |
| Ag1 | 500 | 10.3 | 2.0 | 1.2 | 20.5 | 31.2 |
| | 0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Genway HBsAg ayw | 1000 | 16.4 | 1.3 | 0.8 | 44.5 | 41.3 |
| Ag2 | 500 | 8.5 | 1.1 | 0.9 | 22.2 | 22.2 |
| | 0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Genway HBsAg adw | 1000 | 2.2 | 1.1 | 0.5 | 8.2 | 11.5 |
| Ag3 | 500 | 1.6 | 1.1 | 0.7 | 5.3 | 7.4 |
| | 0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Acris HBsAg ad | 1000 | 4.6 | 3.0 | 0.1 | 16.8 | 26.2 |
| Ag4 | 500 | 2.9 | 2.0 | 0.3 | 11.5 | 16.0 |
| | 0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Acris HBsAg ay | 1000 | 1.9 | 3.1 | 3.4 | 24.0 | 4.5 |
| Ag5 | 500 | 1.2 | 2.0 | 1.0 | 13.2 | 2.4 |
| | 0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

**Table 3: Capture antibodies combination screening (MTP)**

| | | Detection AP | | |
|---|---|---|---|---|
| **Capture Antigen** | Anti-HBsAg [mIU/mL] | Acris ad/ay | Acris HBsAg Ay | Acris HBsAg ad |
| | | Modulation | Modulation | Modulation |
| Cell Sciences HBsAg adw | 1000 | 22 | 17 | 14 |
| Genway HBsAg Ayw | 500 | 11 | 9 | 7 |
| | 100 | 3 | 3 | 2 |
| | 0 | 1 | 1 | 1 |

## 2.2 Detector Stabilizers

In order to improve the signal/background ratio, the effect of four detector diluents, 3% BSA blocking buffer, in-house Alkaline phosphatase stabilizer, biostab, and stabilzyme was tested. Of the four detector diluents, stabilzyme showed the best modulation. Therefore, stabilzyme was finalized as the detector stabilizer.

**Table 4: Detector stabilizers comparison**

| | Assay Buffer (Blocking Buffer) | | | In-House AP stablizerBlocking Buffer | | | Biostab Buffer | | | Stabilzyme Buffer | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WHO [mIU/mL] | Mean | CV% | S/B | Mean | CV% | S/B | Mean | CV% | S/B | Mean | CV% | S/B |
| 1000 | 12786 | 23 | 29.5 | 19073 | 29 | 29.1 | 5848 | 30 | 21.6 | 22972 | 10 | 38.6 |
| 250 | 3417 | 8 | 7.9 | 7054 | 14 | 10.8 | 1755 | 49 | 6.5 | 5559 | 10 | 9.3 |
| 0 | 433 | 24 | 1.0 | 655 | 20 | 1.0 | 271 | 15 | 1.0 | 595 | 12 | 1.0 |

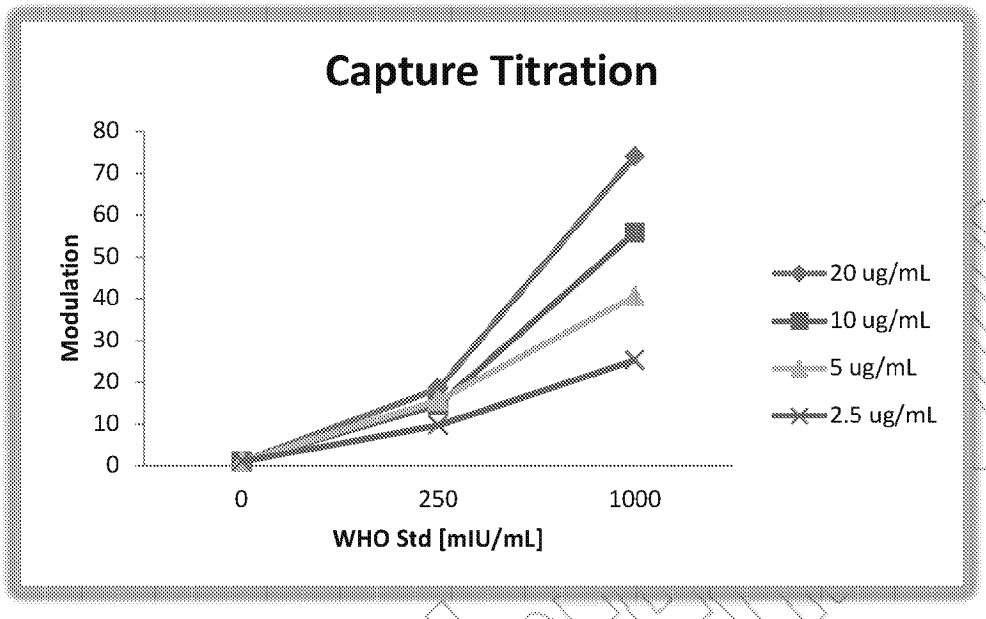
## 2.3 Capture Surface Titration

To optimize the surface, HBs surface antigen titration was performed. Tips were coated with capture antigen cag1/cag2 at 20, 10, 5.0 and2.5 ug/ml. The assay was performed using a 5X_co-incubation_5_5 min protocol on the Theranos system. Sample dilution was 1:5. Detector at 100 ng/ml was prepared in stabilzyme. Capture surface at 5 ug/ml gave an acceptable modulation compared to 20 ug/mL and 10 ug/mL. Hence capture surface at 5 ug/mL was chosen as the final condition.

**Table 5: Capture surface titration**

| | Capture 20 ug/mL | | | Capture 10 ug/mL | | | Capture 5.0 ug/mL | | | Capture 2.5 ug/mL | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WHO [mIU/mL] | Mean | CV% | S/B | Mean | CV% | S/B | Mean | CV% | S/B | Mean | CV% | S/B |
| 1000 | 115733 | 4 | 74.1 | 107105 | 14 | 55.8 | 66973 | 27 | 40.6 | 56758 | 12 | 25.3 |
| 250 | 28947 | 12 | 18.5 | 28109 | 4 | 14.6 | 25808 | 7 | 15.7 | 21844 | 9 | 9.7 |
| 0 | 1563 | 9 | 1.0 | 1921 | 15 | 1.0 | 1648 | 24 | 1.0 | 2242 | 15 | 1.0 |

**Figure 2: Capture surface titration**



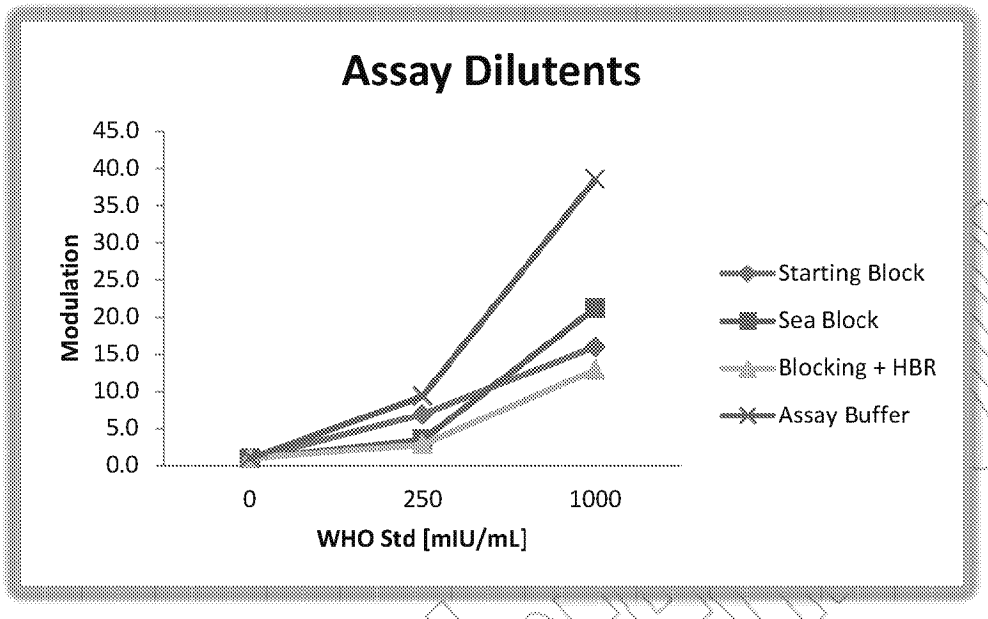

## 2.4 Effect of Assay Diluents

In this experiment, four different assay diluents were tested: assay buffer (3% BSA), starting block, sea block, and assay buffer + HBR. The protocol is 5X_co-incubation_5_5 minute. Sample dilution was 1:5. Detector at 100 ng/ml in stabilzyme was used. The results displayed that assay buffer 3% BSA has the best performance.

**Table 6: Effect of Assay Diluents**

| | Starting block | | | Sea block | | | Blocking + HBR | | | Assay buffer | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WHO [mIU/mL] | Mean | CV% | S/B | Mean | CV% | S/B | Mean | CV% | S/B | Mean | CV% | S/B |
| 1000 | 6438 | 18 | 16.0 | 11085 | 16 | 21.2 | 12843 | 25 | 12.9 | 22972 | 10 | 38.6 |
| 250 | 2778 | 30 | 6.9 | 1831 | 25 | 3.5 | 2862 | 8 | 2.9 | 5559 | 10 | 9.3 |
| 0 | 402 | 14 | 1.0 | 523 | 27 | 1.0 | 997 | 24 | 1.0 | 595 | 12 | 1.0 |

**Figure 3: Assay diluents**



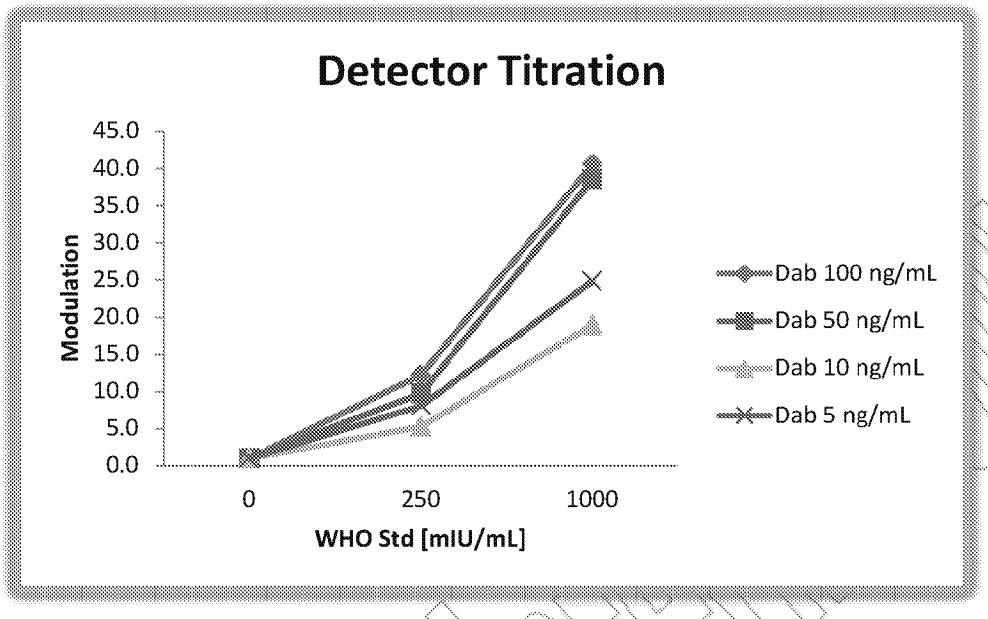

## 2.5 Detector Titration

AP_labeled-HBs Surface antigens were titrated at 5, 10, 50, 100 ng/mL to determine the optimal concentration. The protocol is 5X_co-incubation_5_5 minute. Sample dilution was 1:5. Stabilzyme was used as the diluent. Four concentrations of detector were tested and detector at 100 ng/ml in stabilzyme produced high signals and best modulations. 50 ng/mL also seems to be acceptable.

**Table 7: Detector titration**

| | Final Dab 100 ng/mL | | | Final Dab 50 ng/mL | | | Final Dab 10 ng/mL | | | Final Dab 5 ng/mL | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WHO [mIU/mL] | Mean | CV% | S/B | Mean | CV% | S/B | Mean | CV% | S/B | Mean | CV% | S/B |
| 1000 | 170007 | 19 | 40.6 | 90276 | 9 | 38.5 | 16532 | 12 | 18.9 | 10569 | 10 | 24.9 |
| 250 | 51126 | 15 | 12.2 | 22765 | 14 | 9.7 | 4605 | 11 | 5.3 | 3428 | 17 | 8.1 |
| 0 | 4191 | 26 | 1.0 | 2343 | 18 | 1.0 | 874 | 3 | 1.0 | 425 | 11 | 1.0 |

**Figure 4: Detector titration**




## 2.6 Sample Dilution

Two different sample dilutions, 5 and 10 times were tested to further optimizing the assay. 10 times sample dilution did not produce better signal response than 5 times sample dilution. Hence 5x sample dilution was finalized.

**Table 8: Sample Dilution**

| | 5x coincubation_5_5 | | | 10x coincubation_5_5 | | |
|---|---|---|---|---|---|---|
| WHO [mIU/mL] | Mean | CV% | Modulation | Mean | CV% | Modulation |
| 1000 | 134779 | 9 | 76.7 | 105320 | 23 | 58.4 |
| 500 | 61482 | 30 | 35.0 | 51953 | 20 | 28.8 |
| 250 | 29787 | 22 | 17.0 | 27792 | 15 | 15.4 |
| 100 | 12928 | 23 | 7.4 | 13739 | 6 | 7.6 |
| 50 | 7674 | 23 | 4.4 | 5441 | 14 | 3.0 |
| 10 | 3924 | 6 | 2.2 | 2863 | 10 | 1.6 |
| 0 | 1757 | 17 | 1.0 | 1803 | 16 | 1.0 |

## 2.7 Protocol Test

In order to efficiently evaluteting the assay, Five protocols had been tested, 10X_PSW_5-5-5 min, 10X_PSW_10-10-10 min, 5X_PSW_10-10-10 min, 5X_co-incubation_5_5 min and 10X_ coincubation_5_5. Overall co-incubation format is better option for anti-HBs assay. The co-incubation protocol chose to be a final format.

**Table 9: Protocol Test**

| | 10x_PSW_5-5-5 | | | 10x_PSW_10-10-10 | | | 5x_PSW_10-10-10 | | |
|---|---|---|---|---|---|---|---|---|---|
| WHO [mIU/mL] | Mean | CV% | Modulation | Mean | CV% | Modulation | Mean | CV% | Modulation |
| 1000 | 29521 | 15 | 4.4 | 24979 | 12 | 5.4 | 29521 | 15 | 4.4 |
| 500 | 20506 | 26 | 3.1 | 15879 | 17 | 3.5 | 20506 | 26 | 3.1 |
| 250 | 11387 | 22 | 1.7 | 11033 | 13 | 2.4 | 11387 | 22 | 1.7 |
| 100 | 9669 | 23 | 1.5 | 7811 | 14 | 1.7 | 9669 | 23 | 1.5 |
| 50 | 6477 | 23 | 1.0 | 7198 | 12 | 1.6 | 6477 | 23 | 1.0 |
| 0 | 6663 | 9 | 1.0 | 4600 | 7 | 1.0 | 6663 | 9 | 1.0 |

| | 5x_coincubation_5_5 | | | 10x_coincubation_5_5 | | |
|---|---|---|---|---|---|---|
| WHO [mIU/mL] | Mean | CV% | Modulation | Mean | CV% | Modulation |
| 1000 | 134779 | 9 | 76.7 | 105320 | 23 | 58.4 |
| 500 | 61482 | 30 | 35.0 | 51953 | 20 | 28.8 |
| 250 | 29787 | 22 | 17.0 | 27792 | 15 | 15.4 |
| 100 | 12928 | 23 | 7.4 | 13739 | 6 | 7.6 |
| 50 | 7674 | 23 | 4.4 | 5441 | 14 | 3.0 |
| 10 | 3924 | 6 | 2.2 | 2863 | 10 | 1.6 |
| 0 | 1757 | 17 | 1.0 | 1803 | 16 | 1.0 |

## 2.8 Standard Curve

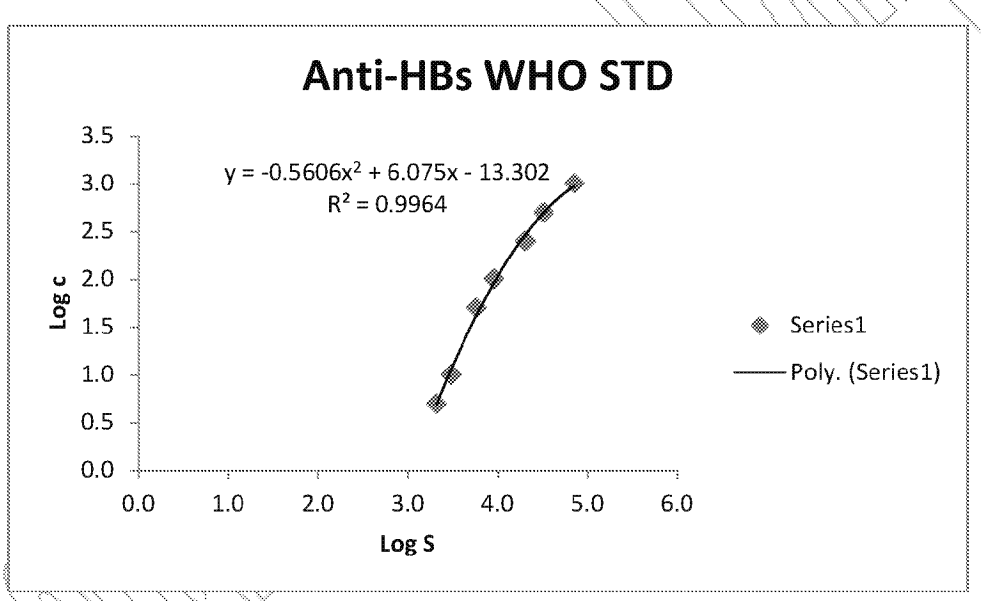
A standard curve ranging from 1000 – 5 mIU/mL was prepared from the WHO Cat # 07/164 was run to determine the assay range. The calibrators were verified on the Siemens Immulite (Cat# L2KAB2) test to confirm. The analyte level of ≥10mIU/mL will be reported as positive on the Theranos assay.

**Table 10: Standard Cruve determination**

| Nominal Sample [mIU/mL] | Assigned from Siemens [mIU/mL] | Avg. RLU | CV% | Modulation | Backcalculated [mIU/mL] | % Recovery |
|---|---|---|---|---|---|---|
| **1000** | **837** | **72200** | **25** | **46.9** | **956** | **114** |

| | | | | | | |
|---|---|---|---|---|---|---|
| 500 | 406 | 32712 | 23 | 21.2 | 499 | 123 |
| 250 | 206 | 20521 | 13 | 13.3 | 295 | 143 |
| 100 | 86.3 | 9131 | 19 | 5.9 | 92 | 107 |
| 50 | 42.7 | 5859 | 32 | 3.8 | 43 | 100 |
| 10 | 10.1 | 3065 | 14 | 2.0 | 12 | 115 |
| 5 | | 2084 | 22 | 1.4 | 5 | |
| 0 | 0 | 1541 | 18 | 1.0 | | |

**Figure 5: Standard cruve graph**




## 2.9 Cross-reactivity

Assay cross-reactivity was determined by testing a number of WHO QC samples, Rheumatoid factor and HAMA positive serum/plasma. 3 out of 22 samples tested showed up as positive on the Theranos assay. These samples also tested positive in the reference assays. Three samples were tested positive also showing positive on the reference assays. The value cut-off is ≥10 mIU/mL is reported positive.

**Table 11: Cross-reactivity**

| QC Controls and samples | Average RLU | Back-calculated [mIU/mL] |
|---|---|---|
| Anti-HBc QC1 Total | 947 | 0.4 |
| Anti-HBc QC2 Total | 645 | 0.2 |
| Anti-HBe QC 1 | 815 | 0.3 |
| Anti-HAV QC1 Total | 842 | 0.4 |
| Anti-HSV1 QC1 | 24408 | 220.8 |
| Anti-HCV Abnova | 750 | 0.2 |

| | | |
|---|---|---|
| Anti-CMV QC1 | 1134 | 0.7 |
| Anti-HIV-1 QC2 | 1525 | 1.4 |
| Anti-HIV-1 QC3 | 8470 | 48.6 |
| Anti-HIV-2 QC2 | 2332 | 4.5 |
| HAMA H20 | 1823 | 2.2 |
| HAMA H21 | 733 | 0.2 |
| HAMA H22 | 746 | 0.2 |
| HAMA H23 | 1485 | 1.5 |
| HAMA H24 | 766 | 0.3 |
| HAMA H25 | 12021 | 84.3 |
| Rf R18 | 1084 | 0.6 |
| Rf R19 | 563 | 0.1 |
| Rf R20 | 716 | 0.1 |
| Rf R21 | 794 | 0.3 |
| Rf R22 | 692 | 0.2 |
| Rf R23 | 677 | 0.2 |

**Table 12: Confirmation of samples on different assay**

| QC Controls samples | Biorad Kit | Siemens mIU/mL |
|---|---|---|
| Anti-HSV1 QC1 | Positive | 467 |
| Anti-HIV-1 QC3 | Positive | 684 |
| HAMA H25 | Positive | >1000 |

## 2.10 Normals Screening

In order to further evaluate the performance of the assay, thirty normal plasma samples from the Stanford blood bank were randomly chosen to compare on the Theranos assay, Siemens Immulite and Biorad ELISA kit. The results for the Theranos assay tracked well with the Siemens Immulite and Biorad results. The range of the standard curve used to calculate the result is from 0, 5, 10, 50, 100, 250, 500, 1000 mIU/mL (Table 9). The analyte level of ≥10mIU/mL will be reported as positive on the Theranos assay.

**Table 13: Normals samples**

| | mIU/mL | | BioRad Kit Result |
|---|---|---|---|
| Samples Normals | Siemens | Theranos | Qualitative |
| M2 | >1000 | 880 | Positive |
| F3 | >1000 | 630 | Positive |

| | | | |
|---|---|---|---|
| F4 | 6.0 | 11 | Negative |
| M5 | 41.4 | 52 | Negative |
| M6 | <3.0 | 1 | Negative |
| M9 | >1000 | 241 | Positive |
| F10 | 75.4 | 617 | Positive |
| M15 | 53.4 | 580 | Positive |
| M14 | 529 | 1260 | Positive |
| F13 | 30.6 | 161 | Positive |
| M12 | < 3.0 | 0.30 | Negative |
| M11 | 4.54 | 6 | Negative |
| M10 | 60.3 | 4 | Negative |
| M91 | <3.0 | 1 | Negative |
| M8 | 459 | 1400 | Positive |
| F8 | 13.3 | 9 | Negative |
| 62 | < 3.00 | 66 | Negative |
| 64 | < 3.00 | 0.37 | Negative |
| 65 | < 3.00 | 0.23 | Negative |
| 77 | < 3.00 | 0.45 | Negative |
| 78 | 51.8 | 3.95 | Positive |
| 79 | 22.1 | 2.21 | Positive |
| 80 | >1000 | 1409 | Positive |
| 81 | <3.00 | 0.40 | Negative |
| 82 | <3.00 | 0.19 | Negative |
| 83 | <3.00 | 0.37 | Negative |
| 85 | <3.00 | 0.22 | Negative |
| 86 | <3.00 | 2.13 | Negative |
| 89 | 24.5 | 580 | Negative |
| 90 | >1000 | 1013 | Positive |

## 2.11 Clinical Samples

The specificity and accuracy of this assay was evaluated by testing three different anti-HBs panels from commercial sources. These are SeroDetect Anti-HBs panel (Cat # K-ZMC008), SeraCare Anti-HBc/HBs Mixed titer performance panel (Cat # PHG203(M2)) and ZeptoMetrix Hepatitis B Panel (Cat # HBV 11000). All these panels have reported values for Anti-HBs antibodies from low to high. The value cut-off is ≥10 mIU/mL is reported positive. The value is ≤10 mIU/mL is non-reactive.

A) There are six panel members in the SeroDetect Anti-HBs Panel. The samples vary in reactivity from negative to high positive. The Theranos assay showed a response trend consistent with DiaSorin ETI-AB-AUK PLUS.

**Table 14: SeroDetect Anti-HBs Panel**

| Panel Member | Theranos mIU/mL | DiaSorin ETI-AB-AUK PLUS (Anti-HBs)(S/Co) |
|---|---|---|
| 1 | 0.6 | 0.03 |
| 2 | 2.0 | 4 |
| 3 | 4.0 | 7.08 |
| 4 | 18.1 | 18.67 |
| 5 | 96.1 | >25.00 |
| 6 | 180.6 | >25.00 |

B) Anti-HBc/HBs mixed titer performance panel from SeraCare has twelve samples. It is mix of samples with varying reactivity. The Theranos assay tracks well with Abbott Anti-HBs Architect.

**Table 15: Anti-HBc/HBs Mixed Titer Performance Panel (Modified)**

| Member ID# | Theranos mIU/mL | Abbott Anti-HBs Architect mIU/mL |
|---|---|---|
| PHG203-01 | 0.8 | 13 |
| PHG203-04 | 1363.0 | >1000.0 |
| PHG203-05 | 901.1 | >1000.0 |
| PHG203-06 | 2.0 | 2.8 |
| PHG203-07 | 1377.3 | 687.5 |
| PHG203-08 | 7.8 | 20 |
| PHG203-09 | 4.1 | 51 |
| PHG203-10 | 203.3 | 175 |
| PHG203-11 | 2.7 | 0.4 |
| PHG203-12 | 0.3 | 0.2 |
| PHG203-13 | 3.6 | 0.6 |
| PHG203-14 | 0.7 | 0.7 |

C) The ZeptoMetrix Hepatitis B panel has a series of nine samples collected from one donor through different dates these samples show decreasing levels of anti-HBs.

**Table 16: ZeptoMetrix Hepatitis B Panel**

| Panel Member | Theranos | Abbott Anti-HBs Architect |
|---|---|---|

| | mIU/mL | mIU/mL |
|---|---|---|
| 11000-01 | 8.3 | 26.4 |
| 11000-02 | 6.0 | 20.2 |
| 11000-03 | 7.1 | 12.4 |
| 11000-04 | 1.2 | 10.2 |
| 11000-05 | 2.9 | 7.4 |
| 11000-06 | 0.7 | 5.2 |
| 11000-07 | 0.7 | 1.8 |
| 11000-08 | 0.4 | 0.7 |
| 11000-09 | 0.5 | 0.5 |

## References


1. Siemens Immulite 2000 anti-HBc IgM assay

2. Bio-Rad MONOLISA ANTI-HBs ELISA kit