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Hepatitis B Surface Antigen Assay Report

Theranos, Inc.

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

1.1 Assay Specifications[TC "Assay Specifications" \f C \l "3"]

Hepatitis B virus (HBV) infection is the 10th leading cause of death worldwide. The most important diagnostic and screening marker for HBV infection is Hepatitis B surface antigen (HBsAg), also known as Australia antigen. It indicates hepatitis B infection.

During infection, HBV produces an excess amount of hepatitis B surface antigen (HBsAg), which can be detected in the blood of infected individuals. HBsAg is the first serological marker after infection with HBV appearing one to ten weeks after exposure and two to eight weeks before the onset of the hepatitis. HBsAg persists during this acute phase and clears late in the convalescence period. Failure to clear HBsAg within six months indicates a chronic HBsAg carrier state. Patients who have vaccinated will develop antibodies against HBsAg (anti-HBsAg seroconversion) are usually considered non-infectious.

This assay is designed to qualitatively determine the presence of surface antigens to Hepatitis B Virus (HBV) in human serum and plasma.

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

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

- Bio-Rad GS HBsAg EIA 3.0 (Cat #25258)
- Siemens Immulite 2000 HBsAg (Cat# L2KHB2)

1.3 Materials and Methods[TC "Materials and Methods" \f C \l "1"]

HBsAg assay format is designed as a sandwich ELISA. The capture surface consists of two biotin-labeled anti-HBsAg antibodies coated on an avidin surface. The sample (plasma or serum) is diluted and incubated for 10 minutes. Then mixed samples will incubate with the detection reagents on the capture surface. Then the surface is washed 8 times and the alkaline phosphatase substrate is incubated on the capture surface for 10 minutes. The resulting chemiluminescence is read in Relative Light Units (RLU) on the Theranos system.

Figure 1: HBsAg Assay Principle

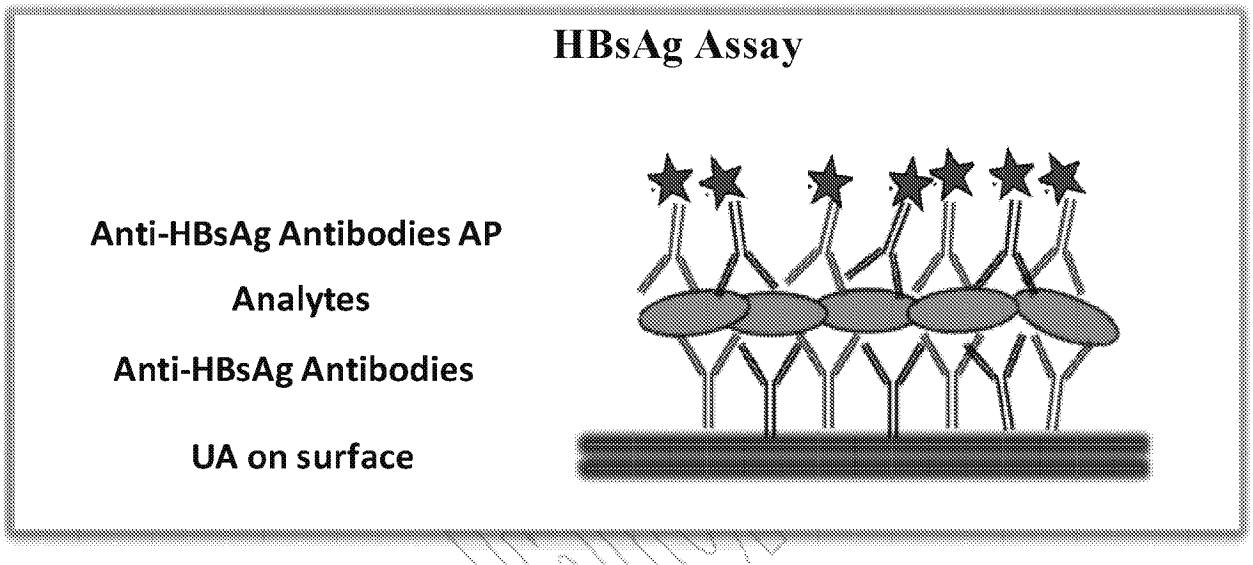

Table 1: Materials

Name	Supplier	Catalog #
HBsAg (adw/ayw) Antibody, Mouse Monoclonal Antibody (Cab7)	Genway	20-511-241567
HEPATITIS B SURFACE ANTIGEN AD/AY (Monoclonal Antibody) (Cab 5)	AbD serotec	4940-3009
WHO International Standard	NIBSC	00/588
HBsAg antibody (Subtype ad/ay) (Dab 37)	Fitzgerald	70-HG15S
HBsAg antibody clone# M8121542 (Dab 40)	Fitzgerald	10-H05B
HBsAg antibody clone# M8121541 (Dab 39)	Fitzgerald	10-H05A
Alkaline Phosphatase Labeling Kit (SH)	Dojindo	LK13
Biotin Labeling Kit (SH)	Dojindo	LK10
Phospho Glo Substrate	KPL	55-60-04
Starting Block Blocking Buffer	Thermo Scientific	37542
Carbonate-bicarbonate buffer	Sigma	C3041
Biostab AP stabilizer	Sigma	76695-250ML-F

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

2. ASSAY DEVELOPMENT[TC "ASSAY OPTIMIZATION" \F C \L "2"]

2.1 Capture Surface: MTP Screen

To determine the best capture surface for the HBsAg assay, 21 Hepatitis B Surface antibodies (Table 2) 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 20ug/mL in carbonate-bicarbonate onto a 384-well plate. Biotinylated Hepatitis B Surface antibodies were added at a concentration of 5ug/ml in blocking buffer. WHO Standard at three levels, 1000, 500, 0 mIU/mL, native and 2 mutants at three levels, 5, 0.5, 0 ng/mL were added as the analyte. AP-conjugated Hepatitis B Surface antibodies at 100ng/ml in blocking buffer were used as detector. The alkaline phosphatase substrate was used to develop chemiluminescent reactions. The resulting RLUs of each of capture surfaces were compared and summarized on Table 3-5 (only showing the highest level). Several Capture antibodies were highly responsive to several detection antibodies. Therefore, they were chosen to combine for further evaluations (Table 6). Furthermore, combo 2 and combo 5 were selected to move forward on to the Theranos system.

Table 2: List of Antibodies Screen on MTP

Antibodies #	Vendors	Cat #
1	Acris	BM3320
2	Acris	BM3029
3	Cosmo	2ZHB16
4	Genway	20-272-192326
5	Abd	4940-3009
6	Genway	20-272-192327
7	Genway	20-511-241567
25	eEnzyme	MHB-312-041
26	eEnzyme	MHB-312-033
36	Fitzgerald	20C-CR2100GP
37	Fitzgerald	70-HG15S
39	Fitzgerald	10-H05A
40	Fitzgerald	10-H05B
41	Fitzgerald	10-H05I
8	Abcam	ab2039
9	Acris	BM3262
10	Novus	NBPI-22888
29	Meridian	G5V18-185
32	Meridian	B035004C
33	Meridian	B01435G
34	Meridian	B65180G

Table 3: 1st Batch Antibodies Screen on MTP

WHO	Dab 1	Dab 2	Dab 3	Dab 4	Dab 5	Dab 6	Dab 7
Cab 1	1.53	1.58	2.00	1.59	1.77	2.08	1.99
Cab 2	1.77	1.39	1.84	1.79	2.15	2.24	2.31
Cab 3	1.75	2.09	1.91	1.75	2.64	2.59	2.31
Cab 4	1.93	2.01	1.45	1.62	2.43	2.21	1.88
Cab 5	1.71	2.44	1.78	1.72	2.00	1.88	1.74
Cab 6	1.38	1.72	1.85	2.49	2.42	2.12	1.69
Cab 7	2.12	1.97	1.92	1.60	1.87	1.90	1.51

Native	Dab 1	Dab 2	Dab 3	Dab 4	Dab 5	Dab 6	Dab 7
Cab 1	1.37	10.49	4.95	4.53	1.71	1.54	2.39
Cab 2	1.68	11.13	10.59	5.90	2.13	2.45	1.95
Cab 3	2.55	16.30	4.71	7.52	4.55	3.83	3.38
Cab 4	1.44	11.05	9.38	5.04	2.00	2.57	2.15
Cab 5	3.95	8.31	4.65	4.65	1.84	1.73	1.54
Cab 6	1.46	7.77	3.97	4.31	1.89	1.80	1.38
Cab 7	1.27	5.84	4.09	3.10	1.63	2.34	1.41

G145R	Dab 1	Dab 2	Dab 3	Dab 4	Dab 5	Dab 6	Dab 7
Cab 1	1.20	1.79	1.20	1.27	1.25	1.06	1.91
Cab 2	0.94	0.66	1.16	1.15	1.66	0.89	1.31
Cab 3	1.44	1.07	1.33	1.07	1.69	1.24	1.05
Cab 4	1.72	1.33	1.11	0.88	1.17	1.12	1.06
Cab 5	1.75	1.32	1.03	1.17	1.06	1.04	1.00
Cab 6	1.32	0.78	1.07	1.23	1.23	1.26	0.97
Cab 7	1.54	0.92	0.99	1.14	1.29	1.14	0.86

Q129H	Dab 1	Dab 2	Dab 3	Dab 4	Dab 5	Dab 6	Dab 7
Cab 1	1.00	1.03	1.47	1.86	1.22	1.03	1.67
Cab 2	0.89	0.77	1.64	1.64	0.73	0.91	1.58
Cab 3	1.04	1.45	1.08	2.32	1.28	1.52	1.63
Cab 4	1.24	1.25	1.88	1.57	1.09	1.20	1.27
Cab 5	0.92	0.99	1.60	1.33	1.03	1.14	1.09
Cab 6	0.74	0.99	1.18	1.07	0.92	1.01	1.18
Cab 7	1.15	1.14	1.13	1.12	1.26	0.88	1.20

Table 4: 2nd Batch Antibodies Screen on MTP

WHO	Dab 25	Dab 26	Dab 36	Dab 37	Dab 39	Dab 40	Dab 41
Cab 25	1.06	1.13	0.93	1.42	1.84	2.43	0.72
Cab 26	0.94	1.63	1.29	1.87	0.89	1.34	0.77
Cab 36	1.47	1.14	1.24	1.67	3.54	1.83	1.10
Cab 37	0.97	1.21	1.50	1.36	3.16	2.05	1.05
Cab 39	1.13	0.94	1.34	1.22	1.37	2.01	1.30
Cab 40	1.12	1.11	1.35	1.18	2.55	2.15	1.00
Cab 41	1.23	1.05	1.34	1.45	1.98	2.28	1.06

Native	Dab 25	Dab 26	Dab 36	Dab 37	Dab 39	Dab 40	Dab 41
Cab 25	0.92	1.17	1.10	15.49	1.66	13.20	4.93
Cab 26	1.04	1.05	1.53	14.36	1.24	7.40	5.20
Cab 36	0.99	1.03	1.62	16.75	1.74	4.36	5.13
Cab 37	1.27	0.91	1.21	2.84	1.17	2.62	1.91
Cab 39	0.73	1.28	1.33	4.05	0.83	2.72	1.76
Cab 40	1.12	1.00	1.17	4.66	1.52	3.46	1.91
Cab 41	1.37	0.79	1.41	1.14	1.42	0.80	1.33

G145R	Dab 25	Dab 26	Dab 36	Dab 37	Dab 39	Dab 40	Dab 41
Cab 25	0.98	1.47	1.04	1.02	0.85	1.28	0.96
Cab 26	1.00	2.67	1.18	1.10	0.98	1.34	1.17
Cab 36	1.15	1.25	1.24	1.22	0.75	1.81	1.73
Cab 37	1.28	1.59	1.18	0.96	0.78	1.36	1.18
Cab 39	1.23	0.90	1.21	1.05	0.95	1.22	1.30
Cab 40	1.11	0.99	1.36	1.36	0.92	1.34	1.35
Cab 41	1.21	1.55	1.36	1.27	0.85	1.09	1.31

Q129H	Dab 25	Dab 26	Dab 36	Dab 37	Dab 39	Dab 40	Dab 41
Cab 25	1.69	0.72	2.27	5.01	2.23	7.54	1.06
Cab 26	1.03	1.72	1.18	3.72	1.04	3.93	1.74
Cab 36	1.41	1.05	1.65	4.77	1.83	2.31	1.88
Cab 37	1.12	1.11	1.07	1.25	0.90	2.03	1.00
Cab 39	1.03	0.85	1.43	1.38	0.65	2.00	0.88
Cab 40	1.11	0.87	0.95	0.83	1.36	1.02	0.52
Cab 41	1.14	0.92	1.66	1.12	0.70	1.34	0.85

Table 5: 3rd Batch Antibodies Screen on MTP

WHO	Dab 4	Dab 5	Dab 10	Dab 29	Dab 32	Dab 33	Dab 34
Cab 4	10.92	0.73	1.03	4.53	5.18	3.78	2.11
Cab 5	4.79	1.97	1.11	4.55	2.94	4.29	1.98
Cab 10	1.70	4.33	1.23	5.40	1.58	7.04	3.89
Cab 29	4.98	3.07	0.94	3.67	1.06	3.30	17.60
Cab 32	14.48	2.52	1.00	3.17	4.53	2.86	0.11
Cab 33	6.45	0.78	1.00	4.15	1.46	2.57	0.34
Cab 34	4.77	2.09	1.12	3.74	2.54	3.08	1.33

Native	Dab 4	Dab 5	Dab 10	Dab 29	Dab 32	Dab 33	Dab 34
Cab 4	4.03	0.41	1.05	6.47	8.66	1.68	1.47
Cab 5	0.82	2.18	1.15	1.01	1.06	1.02	0.93
Cab 10	1.17	4.42	1.26	1.16	1.29	1.56	1.01
Cab 29	2.57	2.54	0.97	5.75	2.91	2.09	15.19
Cab 32	11.02	1.84	1.00	7.13	9.66	1.63	0.08
Cab 33	4.22	0.61	0.99	7.54	3.77	1.57	0.26
Cab 34	2.75	1.89	1.10	4.14	3.78	1.39	1.03

G145R	Dab 4	Dab 5	Dab 10	Dab 29	Dab 32	Dab 33	Dab 34
Cab 4	1.23	0.55	1.05	2.07	2.58	1.39	3.12
Cab 5	1.01	1.71	1.10	0.91	1.00	1.31	0.89
Cab 10	1.52	2.61	1.24	1.46	1.03	2.29	0.68
Cab 29	2.34	2.05	0.96	1.67	1.24	1.81	14.32
Cab 32	4.07	1.95	1.00	1.79	1.57	1.59	0.14
Cab 33	1.74	0.40	0.96	2.01	1.19	1.39	0.25
Cab 34	2.32	2.16	1.10	1.82	2.61	1.40	0.83

Q129H	Dab 4	Dab 5	Dab 10	Dab 29	Dab 32	Dab 33	Dab 34
Cab 4	1.44	0.60	1.04	2.13	2.63	0.70	2.63
Cab 5	1.06	1.34	1.00	0.93	1.01	1.83	0.91
Cab 10	0.96	2.21	1.07	1.09	1.12	1.04	0.94
Cab 29	1.52	1.99	0.92	2.00	1.34	0.65	10.72
Cab 32	3.44	1.58	0.98	1.94	2.35	1.36	0.08
Cab 33	1.45	0.51	0.94	2.17	1.32	0.90	0.51
Cab 34	1.46	1.71	1.07	1.65	1.69	2.23	0.90

Table 6: List of Capture Antibody Combinations Screen on MTP

Combination #	Capture # (5ug/mL)	Detection # (100ng/mL)
1	ab 1 & ab 2	ab 3 & ab 7
2	ab 5 & ab 7	ab 3 & ab 4
3	ab 3 & ab 8	ab 4 & ab 6
4	ab 1 & ab 36	ab 3 & ab 37
5	ab 36 & ab 37	ab 39 & ab 40
6	ab 25 & ab 36	ab 39 & ab 40

Table 7: Capture Antibody Combinations Screen on MTP

Description	Sample	Combo 1	Combo 2	Combo 3	Combo 4	Combo 5	Combo 6
Native, NIBSC 00/588	2 IU/mL	1.0	1.7	1.2	1.6	8.1	0.9
Native, NIBSC 00/588	1 IU/mL	0.9	1.2	1.0	3.9	2.4	1.2
Native, NIBSC 00/588	0 IU/mL	1.0	1.0	1.0	1.0	1.0	1.0
Native HBsAg (ad), Acris BIN142	10 ng/mL	15.5	112.7	42.0	68.7	29.2	6.1
Native HBsAg (ad), Acris BIN142	5 ng/mL	15.7	50.2	22.6	37.3	16.9	3.8
Native HBsAg (ad), Acris BIN142	0 ng/mL	1.0	1.0	1.0	1.0	1.0	1.0
Native HBsAg (ay), MBS318507	10 ng/mL	31.8	22.7	14.9	16.2	16.7	2.1
Native HBsAg (ay), MBS318507	5 ng/mL	15.4	10.4	8.1	7.8	10.7	1.7
Native HBsAg (ay), MBS318507	0 ng/mL	1.0	1.0	1.0	1.0	1.0	1.0
rhHBsAg adr, MBS319180	10 ng/mL	42.1	160.1	23.1	137.8	49.8	9.0
rhHBsAg adr, MBS319180	5 ng/mL	26.8	77.1	12.1	81.8	28.9	5.6
rhHBsAg adr, MBS319180	0 ng/mL	1.0	1.0	1.0	1.0	1.0	1.0
rHBsAg ayw, Genway 10-511-248302	10 ng/mL	12.0	47.8	3.7	7.0	1.2	1.1
rHBsAg ayw, Genway 10-511-248302	5 ng/mL	6.1	22.2	2.0	4.5	1.5	1.0
rHBsAg ayw, Genway 10-511-248302	0 ng/mL	1.0	1.0	1.0	1.0	1.0	1.0
Q129L, MBS14890	10 ng/mL	5.2	17.4	4.4	4.8	3.3	1.7
Q129L, MBS14890	5 ng/mL	2.9	8.2	2.4	2.9	2.2	1.1
Q129L, MBS14890	0 ng/mL	1.0	1.0	1.0	1.0	1.0	1.0
Q129H, Prospec HBS-881	10 ng/mL	21.6	116.8	2.7	1.2	3.5	1.9
Q129H, Prospec HBS-881	5 ng/mL	11.9	54.9	1.8	1.1	2.0	1.5
Q129H, Prospec HBS-881	0 ng/mL	1.0	1.0	1.0	1.0	1.0	1.0
P142S, Prospec HBS-886	10 ng/mL	1.7	3.7	2.3	1.5	3.1	1.2
P142S, Prospec HBS-886	5 ng/mL	1.3	2.2	1.6	1.3	1.4	1.2
P142S, Prospec HBS-886	0 ng/mL	1.0	1.0	1.0	1.0	1.0	1.0
G145R, Genway 10-733-320044	10 ng/mL	1.7	9.9	1.0	0.2	1.4	0.8
G145R, Genway 10-733-320044	5 ng/mL	1.3	5.3	1.0	0.2	1.2	1.0
G145R, Genway 10-733-320044	0 ng/mL	1.0	1.0	1.0	1.0	1.0	1.0

2.2 Three Different Capture Combinations Screen on the Theranos System

To optimize the capture surface, three different capture combinations, combo 2, comb 5, and comb 7 were performed on the Theranos system. Tips were coated with capture antibodies at 5.0ug/ml. The assay was performed using a 5X_co-incubation_10_10 min protocol. Sample dilution was 1:5. Detector combo antibody 39 and antibody 40 at final concentration 100ng/ml was prepared in Biostab. Capture surface Combo 2 gave an acceptable modulation compared to combo 5 and combo 7. Hence combo 2 was chosen as the final pair.

Table 8: Combo 2—Capture Antibody 7 and Capture Antibody 5

Sample	AVG RLU	%CV	Modulation
WHO STD 1 IU/mL	15436	12	16.2
WHO STD 0.250 IU/mL	4538	6	4.8
WHO STD 0.063 IU/mL	1481	10	1.6
0	955	10	1.0
(2) Native HBsAg (ad) 10ng/mL	431760	26	452.3
(6) Native HBsAg (ay) 10ng/mL	84449	6	88.5
(11) rHBsAg (ayw) 10ng/mL	12785	15	13.4
(12) HBsAg (adr) 10ng/mL	164483	4	172.3
(14) K141E 10ng/mL	1050	10	1.1
(18) M133L 10ng/mL	1064	7	1.1
(20) Q129H 10ng/mL	3355	14	3.5
(23) G145R Genway 10ng/mL	2727	24	2.9

Table 9: Combo 5—Capture Antibody 36 and Capture Antibody 37

Sample	AVG RLU	%CV	Modulation
WHO STD 1 IU/mL	12038	11	13.1
WHO STD 0.250 IU/mL	3870	7	4.2
WHO STD 0.063 IU/mL	1375	13	1.5
0	918	11	1.0
(2) Native HBsAg (ad) 10ng/mL	381605	12	415.9
(6) Native HBsAg (ay) 10ng/mL	68648	13	74.8
(11) rHBsAg (ayw) 10ng/mL	10859	17	11.8
(12) HBsAg (adr) 10ng/mL	141802	8	154.5
(14) K141E 10ng/mL	1051	18	1.1
(18) M133L 10ng/mL	1020	8	1.1
(20) Q129H 10ng/mL	3731	22	4.1
(23) G145R Genway 10ng/mL	2991	19	3.3

Table 10: Combo 7—Capture Antibody 29 and Capture Antibody 32

Sample	AVG RLU	%CV	Modulation
WHO STD 1 IU/mL	6007	18	5.1
WHO STD 0.250 IU/mL	4151	15	3.5
WHO STD 0.063 IU/mL	2195	22	1.9
0	1184	16	1.0
(2) Native HBsAg (ad) 10ng/mL	204846	29	172.9
(6) Native HBsAg (ay) 10ng/mL	72619	9	61.3
(11) rHBsAg (ayw) 10ng/mL	13136	28	11.1
(12) HBsAg (adr) 10ng/mL	134178	14	113.3
(14) K141E 10ng/mL	1518	25	1.3
(18) M133L 10ng/mL	2041	11	1.7
(20) Q129H 10ng/mL	3570	20	3.0
(23) G145R Genway 10ng/mL	2769	17	2.3

2.3 Capture Surface Titration

The capture surface combo 2 was titrated at the following concentrations: 10ug/ml, 5ug/ml, 2.5ug/ml, and 1.0ug/ml. WHO standards controls from NIBSC at six levels, 1, 0.5, 0.25, 0.125, 0.063 and 0 IU/mL were used for this screening. The assay was performed using a 5X_co-incubation_10_10 min protocol on the Theranos system. Sample dilution was 1:5. Detector at 500ng/ml was prepared in Biostab, but final concentration was 100ng/mL. The optimal Capture concentration which was determined to be 5ug/ml; gave an acceptable modulation compared to 10ug/mL, 2.5ug/mL, and 1.0ug/mL. Hence capture surface at 5ug/mL was chosen as the final condition.

Table 11: Capture Surface Titration

Cab [ug/mL]	WHO [IU/mL]	AVG RLU	CV	Modulation
1	1	8205	8%	14.3
1	0.5	3807	13%	6.6
1	0.25	1976	13%	3.4
1	0.125	1138	22%	2.0
1	0.063	823	11%	1.4
1	0	575	21%	1.0
2.5	1	10731	42%	13.0
2.5	0.5	5561	26%	6.7
2.5	0.25	2489	13%	3.0
2.5	0.125	2353	6%	2.9
2.5	0.063	1466	12%	1.8

2.5	0	824	15%	1.0
10	1	17836	20%	17.7
10	0.5	6052	43%	6.0
10	0.25	2678	115%	2.7
10	0.125	3110	10%	3.1
10	0.063	1716	6%	1.7
10	0	1006	17%	1.0
5	1	17333	22%	16.4
5	0.5	8704	11%	8.2
5	0.25	4116	9%	3.9
5	0.125	3378	15%	3.2
5	0.063	2307	13%	2.2
5	0	1058	15%	1.0

2.4 Detector Stabilizers

In order to improve the signal/background ratio, the effect of four detector diluents, two commercial alkaline phosphatase stabilizers, biostab and stabilzyme and the 3% blocking buffer were tested as detection antibody (DAb) diluents. Of the four detector diluents, stabilzyme showed the best modulation, but biostab was finalized as the detector stabilizer because the biostab was shown better modulation on the binders and want to keep the same condition as the binders

Table 12: Detector Stabilizers

Conjugate Stabilizer	WHO [IU/mL]	AVG RLU	CV	Modulation
3% BSA Blocking Buffer	1	6012	17%	6.6
3% BSA Blocking Buffer	0.5	2977	18%	3.2
3% BSA Blocking Buffer	0.25	2171	24%	2.4
3% BSA Blocking Buffer	0.125	1536	8%	1.7
3% BSA Blocking Buffer	0.063	1160	19%	1.3
3% BSA Blocking Buffer	0	917	52%	1.0
Biostab	1	17333	22%	16.4
Biostab	0.5	8704	11%	8.2
Biostab	0.25	4116	9%	3.9
Biostab	0.125	3378	15%	3.2
Biostab	0.063	2307	13%	2.2
Biostab	0	1058	15%	1.0
Stabilzyme	1.0	14759	2%	17.4
Stabilzyme	0.5	7319	10%	8.6
Stabilzyme	0.25	4838	12%	5.7

Stabilzyme	0.125	3067	19%	3.6
Stabilzyme	0.063	2117	6%	2.5
Stabilzyme	0	848	11%	1.0

2.5 Detector Titration

AP labeled-Hepatitis B Surface antibodies were titrated at finial concentration 75, 100, 150, and 200 ng/mL which to determine the optimal condition. The protocol is 5X_co-incubation_10_10 minute. Sample dilution was 1:5. Biostab was used as the diluent. The best modulation and high signal between WHO standard controls was observed at 100ng/ml final.

Table 13: Detector Titration

Dab [ng/mL]	WHO [IU/mL]	AVG RLU	CV	Modulation
200	1	19562	13%	10.0
200	0.5	10162	14%	5.2
200	0.25	7289	5%	3.7
200	0.125	3508	7%	1.8
200	0.063	2836	5%	1.5
200	0	1953	14%	1.0
150	1	16232	25%	13.3
150	0.5	11320	4%	9.2
150	0.25	5129	13%	4.2
150	0.125	3569	24%	2.9
150	0.063	2101	6%	1.7
150	0	1225	13%	1.0
75	1	14744	26%	16.8
75	0.5	7725	10%	8.8
75	0.25	4281	9%	4.9
75	0.125	2398	18%	2.7
75	0.063	1360	22%	1.5
75	0	880	7%	1.0
100	1.000	17333	22%	16.4
100	0.500	8704	11%	8.2
100	0.250	4116	9%	3.9
100	0.125	3378	15%	3.2
100	0.063	2307	13%	2.2
100	0.000	1058	15%	1.0

2.6 Detection Antibodies Screen

To further optimize the assay, different detection combinations, including two combinations and three combinations were performed. Tips were coated with three different combination capture antibodies at 5.0ug/ml. The assay was performed using a 5X_co-incubation_10_10 min protocol on the Theranos system. Sample dilution was 1:5. Detector at final concentration 100ng/ml was prepared in Biostab. Capture surface Combo 2 gave an acceptable modulation compared to combo 5 and combo 7. Hence combo 2 and three detection antibodies were chosen as the final pairs.

Table 14: Two Detection Combinations on Capture Combination 2

Combo 2 ab 7/5	Detection combo ab 37/40			Detection combo ab 39/40			Detection combo ab 37/39		
Sample	AVG RLU	%CV	Modulation	AVG RLU	%CV	Modulation	AVG RLU	%CV	Modulation
WHO STD 1 IU/mL	13405	14	14.7	15436	12	16.2	8682	19	9.1
WHO STD 0.250 IU/mL	4848	19	5.3	4538	6	4.8	2913	19	3.1
WHO STD 0.063 IU/mL	1684	9	1.8	1481	10	1.6	1258	9	1.3
0	912	9	1.0	955	10	1.0	949	26	1.0
(2) Native HBsAg (ad) 10ng/mL	572535	4	628.1	431760	26	452.3	461784	6	486.7
(6) Native HBsAg (ay) 10ng/mL	188264	23	206.5	84449	6	88.5	241119	19	254.1
(11) rHBsAg (ayw) 10ng/mL	24164	21	26.5	12785	15	13.4	43711	18	46.1
(12) HBsAg (adr) 10ng/mL	231996	5	254.5	164483	4	172.3	190676	7	200.9
(14) K141E 10ng/mL	1022	15	1.1	1050	10	1.1	1245	2	1.3
(18) M133L 10ng/mL	1514	14	1.7	1064	7	1.1	1976	5	2.1
(20) Q129H 10ng/mL	5991	11	6.6	3355	14	3.5	7108	12	7.5
(23) G145R Genway 10ng/mL	3981	16	4.4	2727	24	2.9	7576	25	8.0

Table 15: Two Detection Combinations on Capture Combination 5

Combo 5 ab 36/37	Detection combo ab 37/40			Detection combo ab 39/40			Detection combo ab 37/39		
Sample	AVG RLU	%CV	Modulation	AVG RLU	%CV	Modulation	AVG RLU	%CV	Modulation
WHO STD 1 IU/mL	20147	13	3.5	12038	11	13.1	15829	1	2.1
WHO STD 0.250 IU/mL	8767	8	1.5	3870	7	4.2	11092	21	1.5
WHO STD 0.063 IU/mL	5686	22	1.0	1375	13	1.5	7471	28	1.0
0	5823	25	1.0	918	11	1.0	7450	11	1.0
(2) Native HBsAg (ad) 10ng/mL	450946	14	77.4	381605	12	415.9	499579	7	67.1
(6) Native HBsAg (ay) 10ng/mL	167563	4	28.8	68648	13	74.8	238731	12	32.0
(11) rHBsAg (ayw) 10ng/mL	25498	4	4.4	10859	17	11.8	42618	13	5.7
(12) HBsAg (adr) 10ng/mL	207051	12	35.6	141802	8	154.5	208107	6	27.9

(14) K141E 10ng/mL	4581	17	0.8	1051	18	1.1	8666	15	1.2
(18) M133L 10ng/mL	6570	18	1.1	1020	8	1.1	7153	21	1.0
(20) Q129H 10ng/mL	10919	15	1.9	3731	22	4.1	14240	15	1.9
(23) G145R Genway 10ng/mL	7987	11	1.4	2991	19	3.3	16673	14	2.2

Table 16: Two Detection Combinations on Capture Combination 7

Combo 7 ab 29/32	Detection combo ab 37/40			Detection combo ab 39/40		
Sample	AVG RLU	%CV	Modulation	AVG RLU	%CV	Modulation
WHO STD 1 IU/mL	10948	13	2.8	6007	18	5.1
WHO STD 0.250 IU/mL	5363	13	1.3	4151	15	3.5
WHO STD 0.063 IU/mL	3643	28	0.9	2195	22	1.9
0	3981	8	1.0	1184	16	1.0
(2) Native HBsAg (ad) 10ng/mL	323671	9	81.3	204846	29	172.9
(6) Native HBsAg (ay) 10ng/mL	89975	11	22.6	72619	9	61.3
(11) rHBsAg (ayw) 10ng/mL	12626	8	3.2	13136	28	11.1
(12) HBsAg (adr) 10ng/mL	147995	8	37.2	134178	14	113.3
(14) K141E 10ng/mL	3552	13	0.9	1518	25	1.3
(18) M133L 10ng/mL	3778	5	0.9	2041	11	1.7
(20) Q129H 10ng/mL	4746	15	1.2	3570	20	3.0
(23) G145R Genway 10ng/mL	5202	5	1.3	2769	17	2.3

Table 17: Three Detection Combinations on Capture Combinations

Detection comb ab 37/ab39/ab 40	Capture Combo 2			Capture Combo 5			Capture Combo 7		
Sample	AVG RLU	%CV	Modulation	AVG RLU	%CV	Modulation	AVG RLU	%CV	Modulation
WHO STD 1 IU/mL	21087	5	14.5	27000	11	2.8	26885	6	2.0
WHO STD 0.250 IU/mL	5829	13	4.0	14026	3	1.4	14630	16	1.1
WHO STD 0.063 IU/mL	2028	12	1.4	10054	23	1.0	14093	9	1.0
0	1456	2	1.0	9723	18	1.0	13732	22	1.0
(2) Native HBsAg (ad) 10ng/mL	625154	14	429.3	589823	6	60.7	601240	11	43.8
(6) Native HBsAg (ay) 10ng/mL	259646	4	178.3	231589	8	23.8	242364	13	17.6
(11) rHBsAg (ayw) 10ng/mL	34361	15	23.6	40627	10	4.2	36271	10	2.6
(12) HBsAg (adr) 10ng/mL	270633	3	185.9	275282	4	28.3	244756	7	17.8
(14) K141E 10ng/mL	1669	8	1.1	10044	25	1.0	12801	9	0.9
(18) M133L 10ng/mL	2267	7	1.6	10565	10	1.1	18386	72	1.3
(20) Q129H 10ng/mL	8817	5	6.1	19196	10	2.0	19504	18	1.4
(23) G145R Genway 10ng/mL	6207	11	4.3	14413	13	1.5	16165	16	1.2

2.7 Effect of Assay Diluents

Four different assay diluents were tested: assay buffer (3% BSA), starting block, sea block, and superblock. The protocol is 5X_co-incubation_10_10 minute. Sample dilution was 1:5. Detector at final concentration 100ng/ml in biostab was used. The results displayed that assay starting block had given best background and high modulation.

Table 18: Assay Diluents

Assay Diluent	WHO [IU/mL]	AVG RLU	CV	Modulation
Seablock	1	1094119	3%	0.9
Seablock	0.5	1046510	10%	0.8
Seablock	0.25	1307575	5%	1.0
Seablock	0.125	1004848	37%	0.8
Seablock	0.063	1028569	14%	0.8
Seablock	0	1271515	16%	1.0
Superblock	1	954800	2%	1.0
Superblock	0.5	966663	5%	1.0
Superblock	0.25	1029491	8%	1.1
Superblock	0.125	725893	15%	0.8
Superblock	0.063	982904	9%	1.0
Superblock	0	954639	9%	1.0
3% BSA	1	21950	12%	6.3
3% BSA	0.5	12461	18%	3.6
3% BSA	0.25	8337	27%	2.4
3% BSA	0.125	8677	25%	2.5
3% BSA	0.063	4630	6%	1.3
3% BSA	0	3500	20%	1.0
Starting Block	1.000	17333	22%	16.4
Starting Block	0.500	8704	11%	8.2
Starting Block	0.250	4116	9%	3.9
Starting Block	0.125	3378	15%	3.2
Starting Block	0.063	2307	13%	2.2
Starting Block	0.000	1058	15%	1.0

2.8 Effect of the Heterophilic Blocking Reagent (HBR)

Both rheumatoid factor (Rf) and Human Anti-Mouse Antibodies (HAMA) positive samples cause false positives in this HBsAg assay on the Theranos system. All samples of the RF samples. Four samples out of the 5 Rf positive samples tested, consistently gave high levels of cross reactivity on the Theranos system. Many assay diluents also tested, but the addition of

HBR in buffer sample diluent was tested in this assay. The result had a significant positive effect and was able to help eliminate false positives. The addition of HBR at the recommended concentration of 400ug/ml was used in this assay. Moreover, the addition of HBR slightly affected the modulation to drop.

Table 19: Comparison of Assay Buffers and HBR

Sample	3% Blocking AVG RLU	Superblock AVG RLU	Sea Block AVG RLU	Surmodics AVG RLU	Starting Block AVG RLU	Starting block HBR AVG RLU
RF 3	44785	116958	147699	144513	44331	1773
RF 4	11845	10986	10896	11847	6234	2177
RF 5	126782	224352	161281	141165	18302	1688

Table 20: HBR Titration

Sample	No HBR AVG. RLU	200ug/mL HBR AVG. RLU	400ug/mL HBR AVG. RLU	1mg/mL HBR AVG. RLU
RF 1	25352	7648	4302	3621
RF 2	4510	4492	2696	4353
RF 3	44331	5057	1621	1773
RF 4	6234	4272	2012	2177
RF 5	18302	4798	1824	1688

2.9 Protocol Screen

In order to efficiently evaluating the assay, serveral protocols had been tested, Generic2_5X_2step_10Pre_Coincubation, Generic2_5X_10Pre_Coincubation, and HBsAg_5X_10Pre_Coincubation_ExtraWash. Overall HBsAg_5X_10Pre_Coincubation_ExtraWash is better option for this assay. It was able to reduce the background, also elimate the false positive on the RF and maintain the decent modulation on the WHO calibrator controls, mutants and natives.

Table 21: Protocol Comparison

	Generic2_5X_2step_10Pre_Coincubation	Generic2_5X_10Pre_Coincubation	HBsAg_5X_10Pre_Coincubation_ExtraWash
Sample	100ng/mL Dab 400ug/mL HBR-1	100ng/mL Dab 400ug/mL HBR-1	100ng/mL Dab 400ug/mL HBR-1
BRH587285	2121	1767	1695
BRH587293	2810	2717	2133
BRH592110	2904	4465	4011
RF A	2517	2324	1443

RF C	2073	2074	1989

2.10 Specificity

Positive disease samples known to cause false positives in the HBsAg assay were tested on the Theranos system. Assay cross-reactivity/interference was determined by testing a number of WHO QC samples, 5 Rheumatoid factors and 5 HAMA positive serums. No cross-reactivity and interference were observed. The value cut-off is ≥1 is reported positive and <1 is negative.

Table 22: Cross-reactivity/Interference

Sample	AVG. RLU	%CV	Theranos [S/Co] 3*SD
HAMA 6	1943	17	0.569
HAMA 7	1972	12	0.577
HAMA 12	1897	11	0.555
HAMA 15	1626	13	0.476
HAMA 16	1726	15	0.505
RF A	1443	20	0.423
RF B	2063	16	0.604
RF C	1989	20	0.583
RF D	2030	21	0.595
RF E	1834	20	0.537
RF F	1815	13	0.532
HAV Biorad	2552	16	0.748
HCV QC1	1942	14	0.569
CMV QC1	2371	24	0.694
EBV	3344	8	0.979
Syphilis	2145	29	0.628
HSV QC1	2350	13	0.688
Toxoplasma QC1	1771	10	0.519
Parvovirus B19	1608	25	0.471
VZV QC1	2828	27	0.828
Rubella QC1	1999	27	0.585

2.11 Determination of the Cutoff

In order to evaluate the performance of the Theranos HBsAg assay, 30 of normal serum/plasma samples were compared among the Theranos and Siemens Immulite. Most samples were tracked well among the 2 assays, although 1 sample out of 30 Siemens Immulite is testing positive. In

addition, 1 sample of 30 in Theranos showed above the cutoff, suggesting that there may be something happened to that sample.

The assay cutoff was determined using the formula Cutoff = AVG RLU (negative samples) + 3*STD. The proposed cutoff RLU was 3414.

Table 23: The Cutoff Determination

Sample	Theranos RLU	Theranos [S/Co] 3*SD	CLIA Lab [S/Co]
BRH587285	1695	0.496	0.582
BRH587286	1928	0.565	1.110
BRH587287	1901	0.557	0.689
BRH587288	1527	0.447	0.634
BRH587289	1718	0.503	0.625
BRH587290	1672	0.490	0.597
BRH587291	1668	0.489	0.571
BRH587292	1434	0.420	0.578
BRH587293	2133	0.625	0.660
BRH587294	1770	0.518	0.684
BRH587295	2097	0.614	0.602
BRH587296	1654	0.484	0.527
BRH587297	1782	0.522	0.583
BRH587298	1792	0.525	0.567
BRH587299	1829	0.536	0.571
BRH587300	1801	0.527	0.589
BRH587301	1846	0.541	0.676
BRH587302	1922	0.563	0.667
BRH587303	3235	0.947	0.631
BRH587304	1779	0.521	0.547
BRH587305	1617	0.473	0.625
BRH587306	1692	0.496	0.609
BRH587307	1768	0.518	0.608
BRH587308	1709	0.500	0.908
BRH592110	4011	1.175	0.597
BRH592111	1709	0.501	0.540
BRH592112	1537	0.450	0.531
BRH592113	1347	0.395	0.621
BRH592114	1725	0.505	0.597
BRH592115	1537	0.450	0.593
Cutoff	**3414**	**1.00**	

2.12 Calibrators/Mutants/Natives

A standard calibrators ranging 2 – 0 IU/mL from WHO cat# 00/588, native antigens (10ng/mL), and mutant antigens (10ng/mL) were run to determine the assay sensitivity. All those calibrators, mutants, and natives also were verified on the Siemens Immulite to confirm. Ther result showed that both assays tracking very well, except that the Theranos system is picked up more mutants thatn the Siemens Immulite. The cutoff is ≥1 will be reported as positive on the Theranos assay and Siemens.

Table 24: Calibrators/Mutants/Natives

WHO Standard [IU/mL]	Theranos AVG. RLU	Theranos [S/Co] 3*SD	CLIA Lab [S/Co]
2.000	29193	8.550	11.600
1.000	14489	4.243	6.350
0.500	8137	2.383	3.510
0.250	4336	1.270	1.950
0.125	3614	1.058	1.250
0.063	2526	0.740	0.800
0.030	2169	0.635	0.773
0.000	2017	0.591	0.523
Native HBsAg (ad)	525355	153.861	102.000
rhHBsAg (adr)	50370	14.752	13.300
rhHBsAg (ayw)	7593	2.224	3.000
Native HBsAg (ad)	233772	68.465	31.100
Native HBsAg (ay)	170282	49.871	12.500
rhHBsAg (adr)	289244	84.711	88.000
rhHBsAg (ad)	68059	19.933	23.400
rhHBsAg (ayw)	1786	0.523	0.504
rHBSAg (adw)	24033	7.038	10.800
rHBSAg (ayw)	13586	3.979	3.980
HBsAg (adr)	121925	35.708	27.400
HBsAg (adw)	6479	1.897	4.710
K-141-E	2016	0.590	0.518
G-145-R	1610	0.472	0.510
M-133-H	1905	0.558	0.506
Q-129-L	5243	1.536	0.728
M133L	2467	0.722	0.568
T126N	2604	0.763	0.588
Q129H	4512	1.321	1.190
P142S	6066	1.777	0.712
T143K	2211	0.648	0.617

G145R Genway	4791	1.403	0.945
D144A	3343	0.979	0.627

2.13 Clinical Samples

The specificity and accuracy of this assay was evaluated by testing five different HBsAg panels from commercial sources. These are SeroDetect HBsAg panel (Cat # K-ZMC007), ZeptoMetrix Hepatitis B Seroconversion Panel (Cat # HBV 6288), ZeptoMetrix Hepatitis B Seroconversion Panel (Cat # HBV 6283), ZeptoMetrix Hepatitis B Seroconversion Panel (Cat # HBV 11000), and ZeptoMetrix Hepatitis B Seroconversion Panel (Cat # HBV 11031). All these panels have reported values for HBsAg from low to high. The value cut-off is ≥1 is reported reactive. The value is <1 is non-reactive.

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

Table 25: SeroDetect HBsAg Panel K-ZMC007

Sample	Theranos Result	DiaSorin ETI-MAK-2 PLUS Result	CLIA Lab Result
Panel Member 1	Non-reactive	Non-reactive	Non-reactive
Panel Member 2	Reactive	Reactive	Reactive
Panel Member 3	Reactive	Reactive	Reactive
Panel Member 4	Reactive	Reactive	Reactive
Panel Member 5	Reactive	Reactive	Reactive

B) The ZeptoMetrix Hepatitis B seroconversion panel cat# HBV 6288 has a series of nine samples collected from one donor through different dates. These samples show increasing level of HBsAg. The Theranos system tracks well with other assays including the CLIA lab.

Table 26: ZeptoMetrix Hepatitis B Seroconversion Panel Cat# HBV 6288

Sample	Theranos Result	ORTHO HBsAg ELISA Testsystem 3	ROCHE Cobas Core HBsAg II EIA	Abbott Murex HBsAg Ver. 3	SORIN ETI-MAK 3	CLIA Lab Result
6288-1	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
6288-2	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
6288-3	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
6288-4	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive

6288-5	Reactive	Reactive	Reactive	Reactive	Non-reactive	Reactive
6288-6	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive
6288-7	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive
6288-8	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive
6288-9	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive

C) The ZeptoMetrix Hepatitis B seroconversion panel cat# HBV 6283 has a series of eleven patient samples collected from one donor through different dates. These samples also show increasing level of HBsAg. The Theranos system tracks well with other assays including the CLIA lab.

Table 27: ZeptoMetrix Hepatitis B Seroconversion Panel Cat# HBV 6283

Sample	Theranos Result	ABBOTT AxSYM HBsAg	ORTHO HBsAg ELISA Testsystem 3	Abbott Murex HBsAg Ver. 3	SORIN ETI-MAK 3	CLIA Lab Result
6283-1	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
6283-2	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
6283-3	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
6283-4	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
6283-5	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
6283-6	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
6283-7	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
6283-8	Reactive	Non-reactive	Reactive	Reactive	Reactive	Reactive
6283-9	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive
6283-10	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive
6283-11	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive

D) The ZeptoMetrix Hepatitis B seroconversion panel cat# HBV 11000 has a series of nine patient samples collected from one donor through different dates. These samples also show increasing level of HBsAg. The Theranos system tracks well with other assays including the CLIA lab, except the Abbott Murex HBsAg Version 3 showing most of samples are reactive.

Table 28: ZeptoMetrix Hepatitis B Seroconversion Panel Cat# HBV 11000

Sample	Theranos Result	ORTHO HBsAg ELISA Testsystem 3	DADE BEHRING Enzygnost HBsAg 5.0	Abbott Murex HBsAg Ver. 3	SORIN ETI-MAK 4	CLIA Lab Result
11000-1	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
11000-2	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
11000-3	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
11000-4	Non-reactive	Non-reactive	Non-reactive	Reactive	Non-reactive	Non-reactive

11000-5	Non-reactive	Non-reactive	Non-reactive	Reactive	Non-reactive	Non-reactive
11000-6	Non-reactive	Non-reactive	Non-reactive	Reactive	Non-reactive	Non-reactive
11000-7	Non-reactive	Reactive	Reactive	Reactive	Reactive	Non-reactive
11000-8	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive
11000-9	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive

E) The ZeptoMetrix Hepatitis B seroconversion panel cat# HBV 11031 has a series of sixteen patient samples collected from one donor through different dates. These samples also show increasing level of HBsAg. The Theranos system tracks well with other assays including the CLIA lab, except the Abbott Prism HBsAg the first two samples and last 8 samples are reactive and only 6 are non-reactive.

Table 29: ZeptoMetrix Hepatitis B Seroconversion Panel Cat# HBV 11031

Sample	Theranos Result	ABBOTT HBsAg Proc C	ORTHO HBsAg Proc B	Abbott Murex HBsAg Ver. 3	Abbott Prism HBsAg	CLIA Lab Result
11031-01	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Reactive	Non-reactive
11031-02	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Reactive	Non-reactive
11031-03	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
11031-04	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
11031-05	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
11031-06	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
11031-07	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
11031-08	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Non-reactive
11031-09	Non-reactive	Non-reactive	Non-reactive	Non-reactive	Reactive	Non-reactive
11031-10	Non-reactive	Non-reactive	Non-reactive	Reactive	Reactive	Non-reactive
11031-11	Reactive	Non-reactive	Non-reactive	Reactive	Reactive	Reactive
11031-12	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive
11031-13	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive
11031-14	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive
11031-15	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive
11031-16	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive