Anti-HBc Total Assay Report

Theranos Inc.

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

1.1 Assay Specifications

Anti-HBc total assay is designed as a Sandwich ELISA to detect total anti-HBc from human blood, plasma or serum from individual patient specimens. This bridging Sandwich ELISA includes HBc antigen as capture and HBc conjugates as detector (Figure 1).

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:

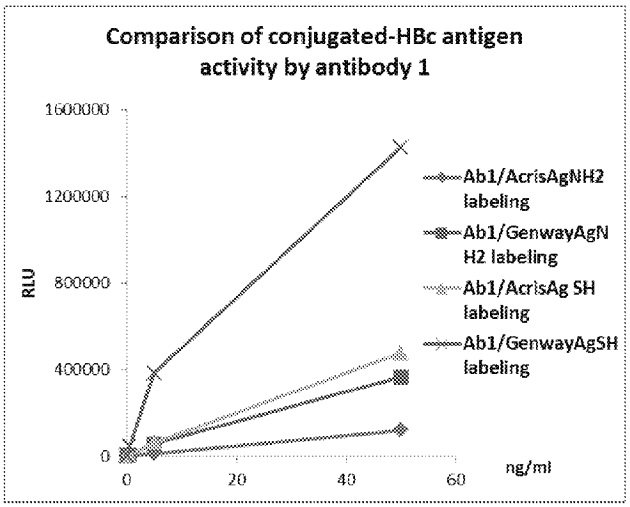
- Bio-Rad Monalisa anti-HBc EIA (Cat #26186)
- Siemens Immulite 2000 anti-HBc (Cat# L2KHC2)

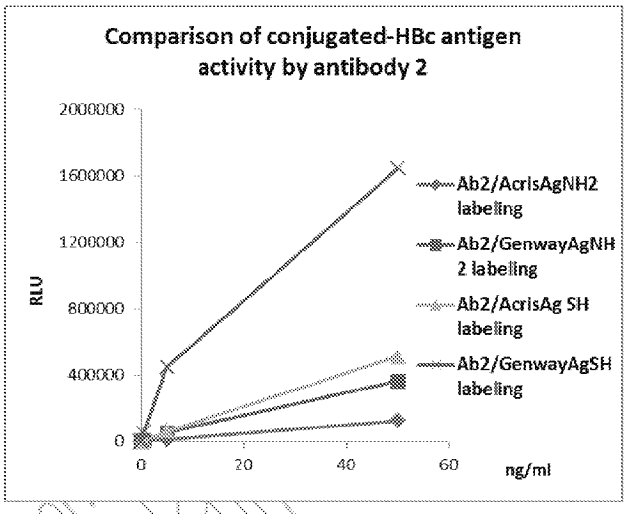
2. ASSAY DEVELOPMENT [TC "Assay Optimization" \f C \l "2"]

2.1 Detector Screening

According to the assay format, HBc antigen conjugates is designed as detector. Two different HBcore proteins derived from E.coli were conjugated with alkaline phosphatase (AP) through either NH2- or SH- residue. Using MTP plate, anti-HBc monoclonal antibodies were directly coated on the plate and AP-HBc conjugates via either NH2 or SH were added. HBc conjugates (Genway) via SH were more reactive to both antibodies (Figure 2). Thus, AP-HBc (Genway) conjugated via SH group was selected as detector.

Figure 2 Test detector's activity (MTP)





2.2 Capture Surface Screening

To determine the optimal capture surface for the Anti-HBc assay, 6 of HBc antigens from different vendors were screened on the Theranos system. The screening was performed with coating each antigen at a concentration of 5 ug/ml in carb-bi-carbobate buffer (pH 9.6). AP-conjugated HBc antigen at 100 ng/ml in 3% BSA-TBS blocking buffer was used as detector. The resulting RLUs of each surface were compared. Capture surface 2 was highly responsive to the selected samples from WHO and commercial ELISA kit controls. Therefore, surface 2 was picked for further evaluations.

Table 1 Capture surface screening

Sample	Surface 1 RLU	Ave. RLU	CV	Surface 2 RLU	Ave. RLU	CV	Surface 3 RLU	Ave. RLU	CV
WHO anti-HBc total	27490	32201	21	23778	28020	21	13193	11774	17
	36912			32262			10354		
QC1 anti-HBc total	9396	10211	11	4038	5530	38	5669	5541	3
	11026			7022			5414		
QC2 anti-HBc total	3163	4484	42	2148	2211	4	2298	2453	9
	5805			2275			2608		
Pooled negative plasma	6529	7048	10	1774	1871	7	1739	1874	10
from 10 negatives (B1)	7567			1968			2009		
Negative plasma individu	1814	2087	18	1375	1499	12	2	1603	#DIV/0!
(B2)	2360			1623			1603		
Biochain P	63174	59119	10	42035	43662	5	21498	25993	24
	55065			45290			30489		
Biochain N	1873	2050	12	1454	1454	#DIV/0!	1439	1602	14
	2227			32			1765		
Biorad P	12884	13427	6	6255	7834	29	9133	8182	16
	13970			9414			7230		
Biorad N	3080	2852	11	1901	1742	13	1957	1759	16
	2625			1584			1561		
	S/B2		15.4			18.7			7.3
	S/B1		4.6			15.0			6.3

Sample	Surface 4 RLU	Ave. RLU	CV	Surface 5 RLU	Ave. RLU	CV	Surface 6 RLU	Ave. RLU	CV
WHO anti-HBc total	10258	15383	47	34093	53178	51	14904	24622	56
	20507			72263			34340		
QC1 anti-HBc total	7693	8206	9	12163	12706	6	5433	5997	13
	8718			13248			6561		
QC2 anti-HBc total	2605	2825	11	13835	9499	65	3991	4806	24
	3045			5163			5620		
Pooled negative plasma	1867	1944	6	5720	6430	16	4150	4349	6
from 10 negatives (B1)	2022			7140			4547		
Negative plasma individu	1386	1562	16	5807	4332	48	1584	1795	17
(B2)	1739			2857			2005		
Biochain P	23476	23814	2	45669	58855	32	53748	53353	1
	24152			72041			52958		
Biochain N	1391	1522	12	1677	1948	20	1317	1614	26
	1652			2219			1910		
Biorad P	9518	9794	4	15721	12313	39	4262	4830	17
	10070			8905			5399		
Biorad N	1826	1765	5	3406	3633	9	2525	2283	15
	1704			3861			2040		
	S/B2		9.8			12.3			13.7
	S/B1		7.9			8.3			5.7

2.3 Capture Surface Titration

In this test, the HBc antigen was titrated at 5, 2.5, 1 and 0.5 ug/ml. Sample dilution is 1:25. Detector at 100 ng/ml was prepared in Stabilizer 1. HBc concentration at 2.5 ug/ml produced the best modulation (signal/background ratio) among all the conditions tested.

Table 2 Capture surface titration

	HBc antigen 5 ug/ml		HBc antigen 2.5 ug/ml		HBc antigen 1 ug/ml		HBc antigen 0.5 ug/ml	
Sample	Ave. RLU	Modulation	Ave. RLU	Modulation	Ave. RLU	Modulation	Ave. RLU	Modulation
WHO anti-HBc total	162042	32.8	140538	34.1	56799	16.1	27837	6.1
WHO anti-HBc QC1	14306	2.9	18179	4.4	18833	5.3	15207	3.3
Pooled negative (B1)	**4937**		**4124**		**3532**		**4574**	
individual #2060	2734		2824		2771		2913	

2.4 Detector Stabilizers

In order to compare whether different stabilizers for detector could improve the signal/background ratio of the positive samples, 2 stabilizers were tested. Stabilizer 1 showed a better modulation. Therefore, stabilizer 1 was locked as detector stabilizer.

Table 3 Detector stabilizers comparison

	Detector 50 ng/ml in Stabilizer 1			Detector 50 ng/ml in Stabilizer 2		
Samples	Ave. RLU	CV	Modulation	Ave. RLU	CV	Modulation
WHO anti-HBc total	57526	5	31.8	29990	4	15.4
WHO anti-HBc QC1	8765	9	4.8	7749	12	4.0
Pooled negative	**1809**	15		**1945**	13	
individual #2060	2128	31		2121	26	

2.5 Detector Titration

In this assay, AP-HBc conjugates were selected as detector. Now, we want to find out at which dose the detector performs the best. Sample dilution is 1:25. Three doses of detector were tested, and detector at 50 ng/ml in stabilizer 1 produced high signals and best modulations.

Table 4 Detector titration

Detector	100 ng/ml		50 ng/ml		25 ng/ml	
Samples	Ave. RLU	Modulation	Ave. RLU	Modulation	Ave. RLU	Modulation
WHO anti-HBc total	140538	34.1	57526	31.8	30390	33.0
WHO anti-HBc QC1	18179	4.4	8765	4.8	5186	5.6
Pooled negative	**4124**		**1809**		**920**	
individual #2060	2824		2128		862	

2.6 Buffer Effects

In this test, two buffers were selected as the coating reagents. Sample dilution is 1:25. Detector at 25 ng/ml was prepared in Stabilizer 1. The results disclosed that coating in carb-bi-carbonate buffer (pH9.6) performed better than coating in PBS buffer.

Table 5 Buffer effects in coating

	Coating in carb-bi-carbonate buffer			Coating in PBS buffer		
Samples	Ave. RLU	CV	Modulation	Ave. RLU	CV	Modulation
WHO anti-HBc total	32624	10	55.5	27309	15	37.2
WHO anti-HBc QC1	3004	11	5.1	862	14	1.2
Pooled negative	**588**	15		**733**	32	
individual #2060	399	11		409	10	

2.7 Protocols Test

Four protocols including different sample dilutions, reagents incubation time and with or without post sample wash (PSW) were compared. The resulting RLUs and modulations revealed that sample dilution at 1:25 with PSW and 5-5-5 min reagents incubation time is the best combined conditions.

Table 6 Protocols test

Sample dilution	1:25		1:25		1:50		1:25	
Incubation time	5-5-5 min		5-5-5 min		5-5-5 min		2-2-1 min	
Post sample wash	Yes		no		Yes		Yes	
Samples	RLU	Modulation	RLU	Modulation	RLU	Modulation	RLU	Modulation
WHO anti-HBc total	30390	33	29149	30.6	17443	16.8	4403	12.9
WHO anti-HBc QC1	5186	5.6	4642	4.9	2670	2.6	621	1.8
Pooled negative	**920**		**954**		**1039**		**342**	
individual #2060	1025		961		1121		409	

2.8 Cross-reactivity

A number of disease samples were tested for the cross-reactivities. All the samples tested showed low RLUs and did not react with the Theranos anti-HBc assay. This suggests that total anti-HBc assay has no cross-reactivity towards the disease samples tested.

Table 7 Cross-reactivity

Sample	RLU1	RLU2	Ave. RLU	%CV
Influenza (US biological kit control)	221	179	204	10
	221	196		
Anti-CMV QC1 (WHO)	352	354	354	27
	471	237		
Anti-HAV QC1 (WHO)	215	453	378	38
	307	538		
Anti-HSV1 QC1 (WHO)	458	327	486	53
	862	298		
Anti-HCV QC1 (WHO)	1593	1591	1498	8
	1467	1340		
Anti-HBs QC1 (WHO)	732	439	478	36
	356	384		
Anti-HBs 10 IU (WHO)	2002	2303	2047	8
	1957	1925		
Anti-HIV 1 QC2 (WHO)	424	725	598	28
	486	758		
Anti-HIV 1 QC3 (WHO)	356	N/A	367	22
	293	451		
Anti-HIV 2 QC2 (WHO)	424	487	429	10
	427	378		
Anti-HIV 2 QC3 (WHO)	1223	1356	1367	8
	1468	1423		
HAMA2	432	522	519	12
	566	557		
HAMA3	427	407	438	13
	519	400		
HAMA4	763	678	593	26
	484	445		
Rheumatoid factor positive 8	417	687	472	31
	385	398		
Rheumatoid factor positive 9	1212	979	1146	13
	1319	1072		
Rheumatoid factor positive 10	698	1127	842	23
	773	772		

2.9 Test Normal Samples

In order to evaluate the performance of the Theranos anti-HBc assay, 20 of normal plasma samples were compared among the Theranos, Siemens Immulite and Biorad ELISA kit. All the specimens showed low RLUs with the Theranos assay, as well as with the other two reference

assays. This indicates that the Theranos ant-HBc assay does not cause false positive reactions with normal samples.

Table 8 Normal samples correlation

Sample IDs	Theranos Ave. RLU	Theranos % CV	Biorad	Siemens (IU)
1	1603	7	0.113	0.463
2	608	33	0.129	0.427
3	765	19	0.181	0.425
4	752	27	0.134	0.491
5	1113	15	0.172	0.479
6	590	3	0.135	0.422
7	535	7	0.126	0.452
8	646	10	0.130	0.478
9	766	23	0.113	0.521
10	1152	31	0.256	0.464
11	717	19	0.202	0.455
12	359	18	0.117	0.41
13	723	23	0.199	0.437
14	570	25	0.298	0.47
15	603	20	0.122	0.515
16	671	27	0.115	0.534
17	702	31	0.120	0.412
18	518	12	0.120	0.435
19	432	16	0.167	0.434
20	572	10	0.096	0.446
Non-R			**0.370**	**<0.85**
R			**0.302**	**>1.15**
cutoff			**0.336**	

2.10 The Cutoff Determination

A total of 36 samples were tested in order to determine the assay cutoff. A proposed cutoff (RLU 2000) was determined based on cutoff = mean+5*SD (standard derivation). **Signal/cutoff (S/co) ratio <1 is defined as anti-HBc non-reactive. S/co ratio > 1 is defined as positive.**

Table 9 The Cutoff determination

Sample IDs	Ave. RLU	% CV	S/co
1	1603	7	0.8
2	608	33	0.3
3	765	19	0.4
4	752	27	0.4
5	1113	15	0.6
6	590	3	0.3
7	535	7	0.3
8	646	10	0.3
9	766	23	0.4
10	1152	31	0.6
11	717	19	0.4
12	359	18	0.2
13	723	23	0.4
14	570	25	0.3
15	603	20	0.3
16	671	27	0.3
17	702	31	0.4
18	518	12	0.3
19	432	16	0.2
20	572	10	0.3
21	501	37	0.3
22	549	21	0.3
23	1566	21	0.8
24	349	11	0.2
25	363	22	0.2
26	561	29	0.3
27	560	17	0.3
28	524	10	0.3
29	553	21	0.3
30	632	24	0.3
31	480	26	0.2
32	589	45	0.3
33	517	15	0.3
34	533	24	0.3
35	585	47	0.3
36	704	48	0.4

2.11 Assay Validation

The specification and accuracy of this assay was verified by different sources of clinical samples with reported anti-HBc values. The SeroDetect Anti-HBcore panel is intended for use with in vitro assay procedures for the determination of antibodies to hepatitis B core antigen. It is composed of five members representing a range of reactivities. The Theranos assay detected that panel #1 is negative and other 4 samples are positive, which is consistent with DiaSorin ETI-COREK report (Table 5). The DiaSorin ETI-COREK assay is a competitive ELISA. As a result, the more anti-HBc antibodies in the samples the lower of the signals generate.

Table 10 Validation results with Zeptometrix SeroDetect anti-HBcore panel

ZeptoMetrix SeroDetect Anti-Hbcore panel	Theranos Anti-HBc (R > 1)			DiaSorin ETI-COREK PLUS (Anti-HBc)
	Ave. RLU	% CV	S/Co	S/Co
Zeptometrix panel #1	358	32	0.2	2.55
Zeptometrix panel #2	31992	14	16.0	0.03
Zeptometrix panel #3	28342	9	14.2	0.01
Zeptometrix panel #4	28105	37	14.1	0.03
Zeptometrix panel #5	34511	17	17.3	0.03

The second panel chosen to monitor our assay performance is Seracare anti-HBc IgM mixed titer performance panel. This panel has 25 naturally occurring plasma specimens with varying levels of reactivity for anti-HBc. Anti-HBc level of each panel members were pre-quantitated with Diasorin ETI-COREK anti-HBc plus assay. Theranos anti-HBc total antibody assay tracked all the clinical samples and the results correlated closely to the Siemens and Diasorin anti-HBc assays.

Table 11 Validation results with Seracare anti-HBc IgM mixed titer panel

Seracare anti-HBc IgM Mixed Titer Performance Panel	Theranos Anti-HBc (R > 1)			Siemens Immulite anti-HBc (R >1.15)	Diasorin ETI-COREK (anti-HBc) Plus
	Ave. RLU	% CV	S/Co	S/Co	S/Co
Seracare 1	347	20	0.2	0.412	0.2
Seracare 2	58469	16	29.2	12.9	132
Seracare 3	70463	11	35.2	17.7	175.3
Seracare 4	61632	8	30.8	23.6	164.4
Seracare 5	49353	8	24.7	24.7	>263
Seracare 6	64393	9	32.2	10.7	76.7
Seracare 7	70216	15	35.1	14.1	175.3
Seracare 8	68611	9	34.3	13.2	197.3
Seracare 9	65202	20	32.6	13.7	197.3
Seracare 10	55476	7	27.7	10.3	197.3
Seracare 11	37945	31	19.0	5.87	197.3
Seracare 12	42331	19	21.2	25.4	197.3
Seracare 13	55677	20	27.8	28.9	>263
Seracare 14	59044	11	29.5	13.7	>263
Seracare 15	58217	5	29.1	14	>263
Seracare 16	67349	26	33.7	35.9	157.8
Seracare 17	58514	17	29.3	26	>263
Seracare 18	43448	9	21.7	21.1	197.3
Seracare 19	72235	17	36.1	17.4	>263
Seracare 20	2539	15	1.3	6.14	43.8
Seracare 21	63181	10	31.6	11.4	>263
Seracare 22	62763	6	31.4	16.5	4.5
Seracare 23	63194	14	31.6	18.9	>263
Seracare 24	78537	8	39.3	13.9	>263
Seracare 25	46123	7	23.1	18.8	>263

The third panel used to evaluate the Theranos anti-HBc total antibody assay is Zeptometrix anti-HBc/anti-Hbs mixed panel. Anti-HBc levels of each panel members were pre-determined with different reference laboratory tests. The Theranos assay is able to detect anti-HBc antibody from most of the specimens in this panel and the result correlates closely to the Bayer ADVIA test.

Table 12 Validation results with ZeptoMetrix anti-HBc/anti-HBs mixed panel

ZeptoMetrix anti-HBc/anti-HBs mixed panel	Theranos Anti-HBc (R > 1)			Bayer Anti-HBc ADVIA Centaur	Ortho anti-HBc Vitros ECI	Abbott anti-HBc EIA
	Ave. RLU	% CV	S/Co	S/Co (R> 1)	Co/S (R>1)	Co/s (R>1)
#1	871	13	0.44	0.4	3.3	2.6
#4	3104	20	1.55	0.9	10	6.7
#5	1661	11	0.83	7.9	10	6.4
#6	12603	13	6.30	>8	10	6.3
#7	790	20	0.39	1.6	1.7	1.0
#8	39783	3	19.89	>8	>10	42.0
#9	583	41	0.29	0.2	0.8	1.4
#10	2292	11	1.15	>8	10	17.9
#11	46618	34	23.31	>8	>10	58.6
#12	52246	9	26.12	>8	>10	75.7