



# **Lyme Disease Antibody (Total IgG and IgM) Assay Development 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" ]

This assay is designed to qualitatively determine the presence of IgG and/or IgM antibodies to Lyme Disease (*Borrelia burgdorferi*) in human serum, plasma or whole blood (automatically processed into plasma by the Theranos System).

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

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

- US Biological Lyme Disease IgG, IgM BioAssay Cat # L8001-22
- Immunetics Lyme Disease IgG, IgM Cat # DK-E352-096

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

A peptide specific to the Lyme disease pathogen serves as the capture surface for Anti-*Borrelia burgdorferi* antibodies in the sample. After incubation of the appropriately-diluted sample on the capture surface, the surface is washed and then a mixture of mouse anti-human IgG and mouse anti-human IgM detection antibodies are incubated on the surface. The surface is washed again. Finally an alkaline phosphatase substrate is incubated on the surface, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

**Table [ SEQ Table \\* ARABIC ]: Materials**

<b>Name</b>	<b>Supplier</b>	<b>Catalog #</b>
C6-25 Biotin Peptide MKK DDQ IAA AIA LRG MAK DGK FAV K	BioSyn	N/A Custom peptide synthesis
Heterophilic Blocking Reagent (HBR)	Scantibodies	3KC533
Mouse Anti-Human IgG Antibody	Novus	NB100-2046
Mouse Anti-Human IgM Antibody	Novus	NB500-468

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

### 1.2 Antibody Screening

The detection antibodies were screened with commercially available Lyme Disease IgG/M Positive and Negative controls on a surface coated with 10 ug/mL of p41 (*Borrelia burgdorferi* flagellin protein) and a sample dilution of 1:25. Since the commercial control does not contain as much Anti-Lyme IgM as IgG, pooled Anti-Lyme positive sera were also used in the screen. All the DABs performed similarly with regards to modulation. IgG DAB #1 and IgM Dab #1 were chosen based on best modulation and past performance in other antibody detection assays.

**Table [ SEQ Table \\* ARABIC ]: Anti-Human IgG and IgM Antibody Information**

Number	Vendor	Cat #	Type
IgG DAb 1	Novus	NB100-2046	MAB
IgG DAb 2	US Biological	I904-75W	MAB
IgG DAb 3	Abcam	ab99761	MAB
IgG DAb 4	Southern Biotech	9042-01	MAB
IgM DAb 1	Novus	NB500-468	MAB
IgM DAb 2	Abd Serotech	5278-5204	MAB
IgM DAb 3	Abd Serotech	5278-5159	MAB
IgM DAb 4	Abd Serotech	MCA1662	MAB

**Table [ SEQ Table \\* ARABIC ]: Anti-IgG DAb Screen**

Dab#	Positive Control		Negative Control		Modulation
	Mean RLU	CV %	Mean RLU	CV %	
IgG Dab 1	1618	15.1	326	24.1	5.0
IgG DAb 2	1101	22.6	290	43.5	3.8
IgG DAb 3	39581	18.3	9187	24.6	4.3
IgG DAb 4	61272	27.6	12692	7.7	4.8

**Table [ SEQ Table \\* ARABIC ]: Anti-IgM DAb Screen**

Dab #	Positive Control		Pooled Positive Sera		Negative Control		Modulation, CTL	Modulation, Pooled Sera
	Mean	CV %	Mean	CV %	Mean	CV %		
IgM DAb 1	12815	15.2	58589	11.8	4832	14.2	2.7	12.1
IgM DAb 2	6150	16.9	20265	31.8	2095	26.6	2.9	9.7
IgM DAb 3	14910	14.7	56363	14.9	5090	16.0	2.9	11.1
IgM DAb 4	38463	24.9	126617	15.7	15170	24.5	2.5	8.3

### 1.3 Antigen Screen

Various *Borrelia burgdorferi* antigens are used in ELISA assays to detect anti-Lyme IgG and IgM, from native antigen derived from sonicated whole organism to recombinant proteins representing different antigenic regions. The antigens were tested with positive and negative controls and a set of positive and negative samples – only samples that were positive in both the US Biological and Immunetics kit were considered “consensus positive” for this purpose, and only samples that were negative in both assays were considered “consensus negative”.

The commercial antigens performed adequately in response to the positive and negative control material, however none of the antigens were able to adequately differentiate positive and negative samples when tested with clinical samples. Various blocking agents were tested as sample diluents and failed to significantly improve differentiation of positive and negative samples with any of the commercial antigens.

Next, a 25 amino acid peptide sequence based on the C6 peptide from the VlsE protein and a 10 amino acid sequence based on the OspC protein were custom synthesized. The C6-25 peptide was able to differentiate positive and negative samples, and with HBR in the diluent showed excellent differentiation. This peptide shows superior specificity and sensitivity for anti-Lyme IgG and IgM according to the most recent research<sup>1,2,3</sup> and our own testing. Furthermore, use of this highly immunogenic but very specific peptide eliminates cross reactivity with individuals who are vaccinated with the recombinant OspA vaccine.

**Table [ SEQ Table \\* ARABIC ]: Antigens Screened**

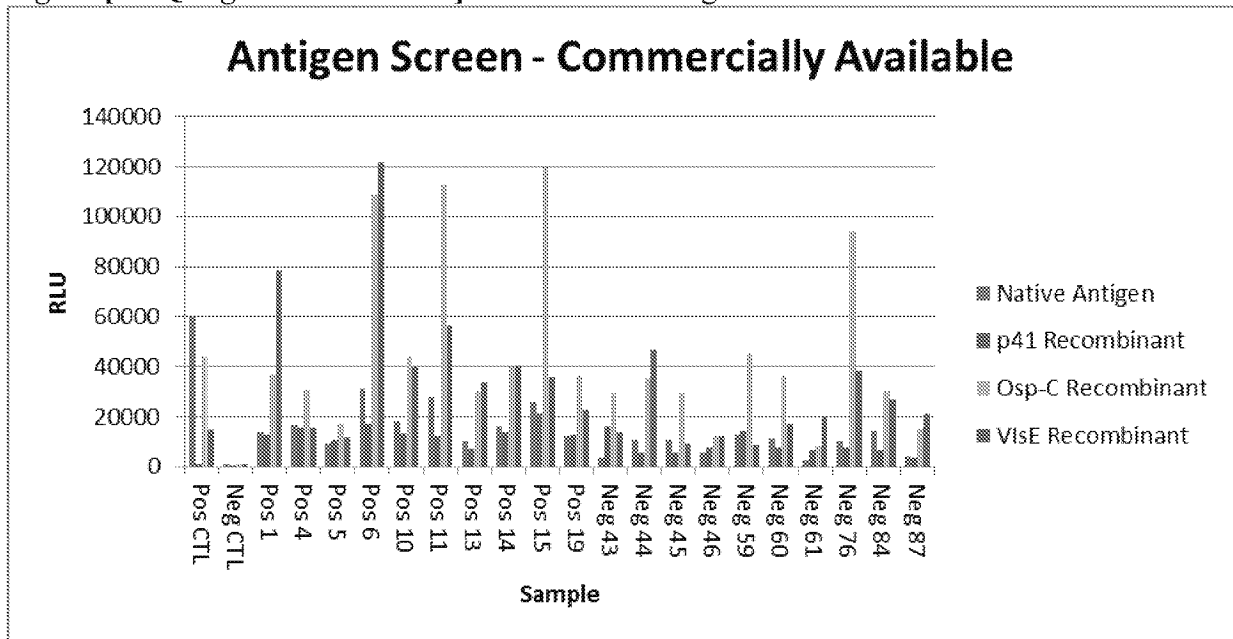
Manufacturer	Cat #	Antigen
Genway	11-783-79721	Native <i>Borrelia burgdorferi</i> , sonicated organisms
US Biological	B2570-26	Flagellin p41 (E.coli derived recombinant)
US Biological	B2570-17A1	Osp-C (Contains a 6-HIS epitope tag)
Bioclone	PP-0128	Outer surface protein VlsE 300aa (E Coli expressed)
BioSyn	Custom	25 amino acid sequence of the C65 peptide
BioSyn	Custom	10 amino acid sequence of the OspC protein



**Table [ SEQ Table \\* ARABIC ]: Screen of Commercial Antigens on a Microtiter Plate**

		Native Antigen	p41 Recombinant	Osp-C Recombinant	ViSE Recombinant
<b>Sample Type</b>	<b>Sample #</b>	<b>RLU</b>	<b>RLU</b>	<b>RLU</b>	<b>RLU</b>
Pos CTL		60135	1229	43963	14790
Neg CTL		886	440	1186	1309
Modulation +/-		68	3	37	11
Positive Samples	1	13702	12582	36976	78398
	4	16849	15627	30508	15437
	5	9096	10810	17278	11568
	6	31324	17139	108898	121971
	10	18039	13302	43638	39585
	11	27957	12060	112764	56612
	13	9960	7329	30127	33501
	14	16279	13753	39741	40205
	15	25933	21206	119489	35752
	19	12349	12667	36307	22693
Pos Mean RLU		18149	13648	57573	45572
Negative Samples	br43	3794	16098	29197	13619
	br44	10563	5748	35385	47039
	br45	10774	5744	29337	9133
	br46	5840	7476	12308	12210
	br59	12799	14344	45564	8529
	br60	11148	7606	36331	17003
	br61	2764	6828	8318	20337
	br76	9905	7744	94153	38457
	br84	13993	6406	30233	26859
	br87	4168	3391	15214	21305
Neg Mean RLU		8575	8139	33604	21449
Modulation Mean Pos/Neg Samples		2	2	2	2

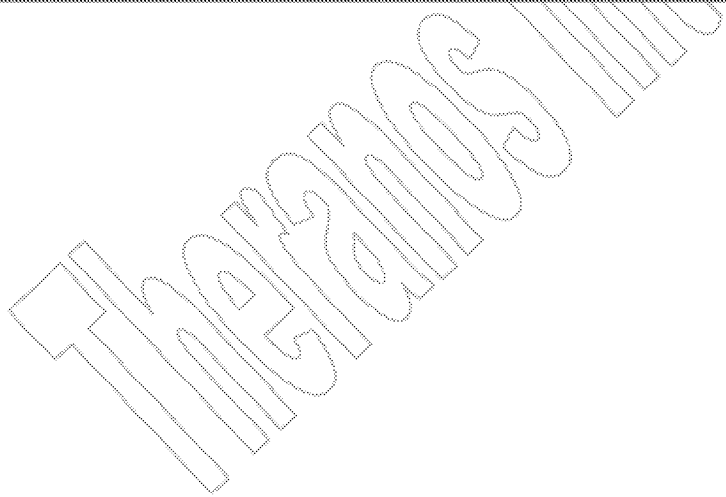
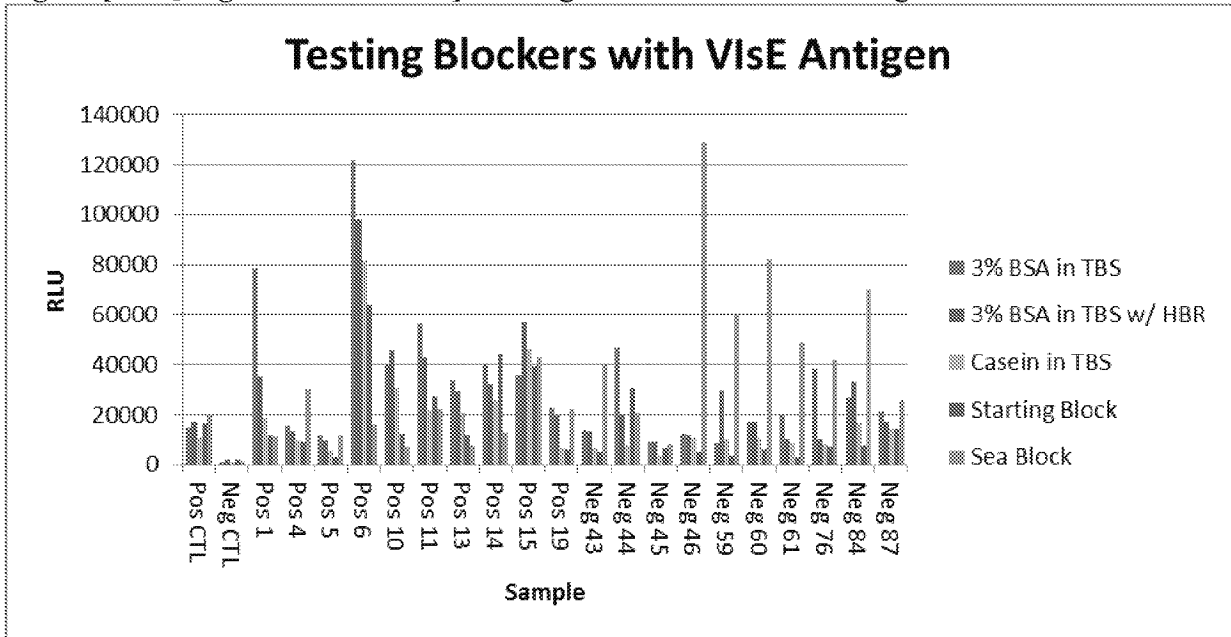
Figure [ SEQ Figure \\* ARABIC ]: Commercial Antigen Screen



**Table [ SEQ Table \\* ARABIC ]:** Testing VisE Antigen with Blockers in Sample Diluent on a Microtiter Plate

Sample Type	Sample #	3% BSA in TBS	3% BSA in TBS w/ HBR	Casein in TBS	Starting Block	Sea Block
		RLU	RLU	RLU	RLU	RLU
Pos CTL		14790	17064	10707	16577	20417
Neg CTL		1309	1936	1152	1938	1823
Modulation +/-		11	9	9	9	11
Positive Samples	1	78398	35139	18559	11786	11303
	4	15437	13005	9767	9060	30295
	5	11568	9726	5658	3015	11769
	6	121971	97883	81497	64058	16117
	10	39585	45842	30856	12331	7087
	11	56612	42791	21893	27360	22128
	13	33501	29251	20897	11847	7832
	14	40205	32405	25615	44196	12533
	15	35752	56636	46410	39114	42995
	19	22693	19493	6476	6334	22025
Pos Mean RLU		45572	38217	26763	22910	18408
Negative Samples	br43	13619	13387	6591	5126	39674
	br44	47039	19969	7446	30531	20908
	br45	9133	9278	3411	6405	8079
	br46	12210	11570	10652	5324	128976
	br59	8529	29683	10192	3485	59811
	br60	17003	17092	10471	6268	82124
	br61	20337	9926	8543	3330	48623
	br76	38457	9882	7963	7034	41903
	br84	26859	33085	16486	7512	69795
	br87	21305	17338	14043	14243	25724
Neg Mean RLU		21449	17121	9580	8926	52562
Modulation Mean Pos/Neg Samples		2	2	2	3	3

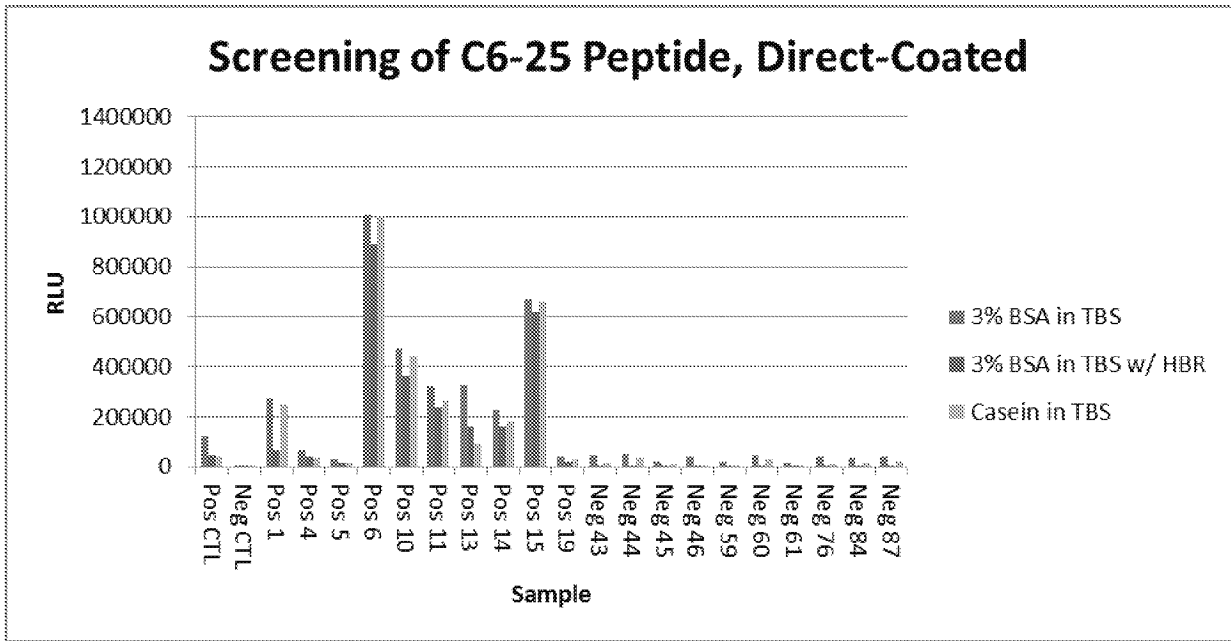
**Figure [ SEQ Figure \\* ARABIC ]:** Testing Blockers with VisE Antigen



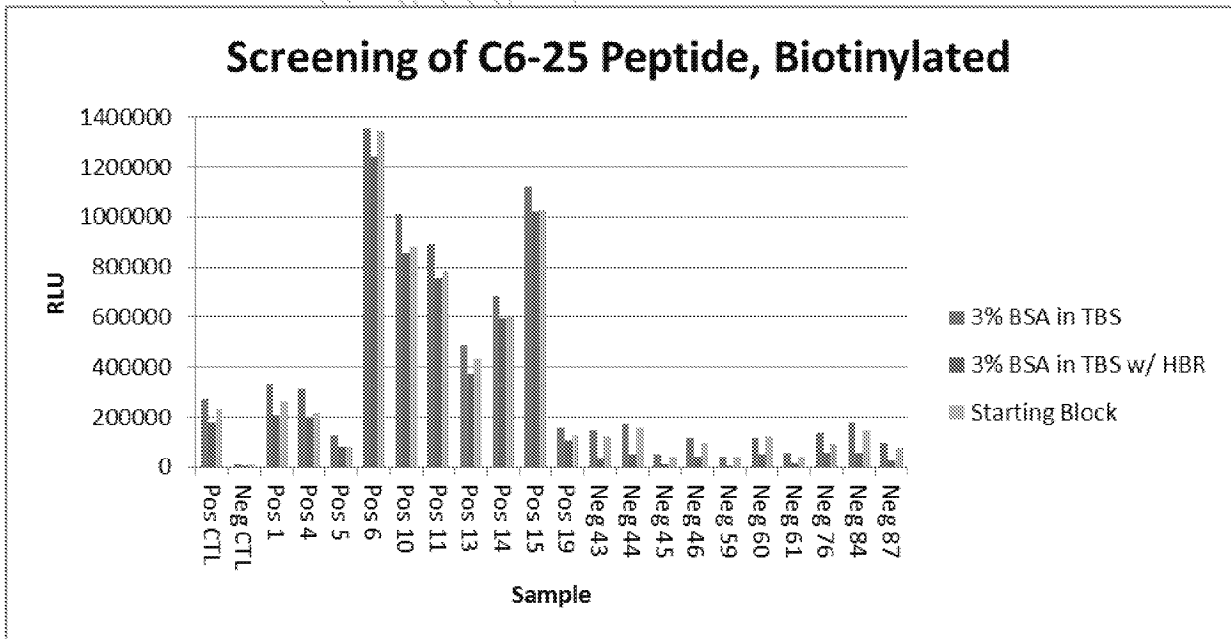
**Table [ SEQ Table \\* ARABIC ]: Screening of C6-25 Custom Peptide on a Microtiter Plate**

Surface:		C6-25 Passive Absorption			C6-25-biotin on Avidin		
Sample Diluent:		3% BSA in TBS	3% BSA in TBS w/ HBR	Casein in TBS	3% BSA in TBS	3% BSA in TBS w/ HBR	Starting Block
Sample Type	Sample #	RLU	RLU	RLU	RLU	RLU	RLU
Pos CTL		123751	44933	40560	274654	175790	231070
Neg CTL		3623	1231	3355	9355	3935	8432
Modulation +/-		34	37	12	29	45	27
Positive Samples	1	271568	63568	248479	331849	205764	263813
	4	68397	42927	35812	311571	196666	215280
	5	29863	14931	17282	127579	82485	78870
	6	1004240	893018	1002881	1351130	1241992	1344985
	10	475721	360515	444422	1012733	856446	880940
	11	322072	239255	261115	888900	757061	785450
	13	327565	159091	91992	488545	373075	434418
	14	227521	160136	180499	684323	592811	597389
	15	668846	620592	661105	1123239	1022039	1027448
	19	42276	22194	31008	157818	106099	126205
Pos Mean RLU		343807	257623	297459	647769	543444	575480
Negative Samples	br43	47687	4922	14387	147232	36919	120889
	br44	49620	3431	34517	169240	51435	154295
	br45	21386	1767	12863	51693	12227	41541
	br46	38966	3146	4820	115198	39730	98137
	br59	22987	2226	7651	40795	8133	38797
	br60	44782	2301	32915	118480	53217	121210
	br61	17472	2375	6280	56213	13404	41523
	br76	38721	2864	12342	134622	54980	93220
	br84	36771	2132	17746	176546	55579	147486
	br87	40325	4590	19501	97027	24263	77261
Neg Mean RLU		35872	2975	16302	110705	34989	93436
Modulation Mean Pos/Neg Samples		10	87	18	6	16	6

**Figure [ SEQ Figure \\* ARABIC ]:** Screening of C6-25 Peptide Direct-Coated with 3 Sample Diluents



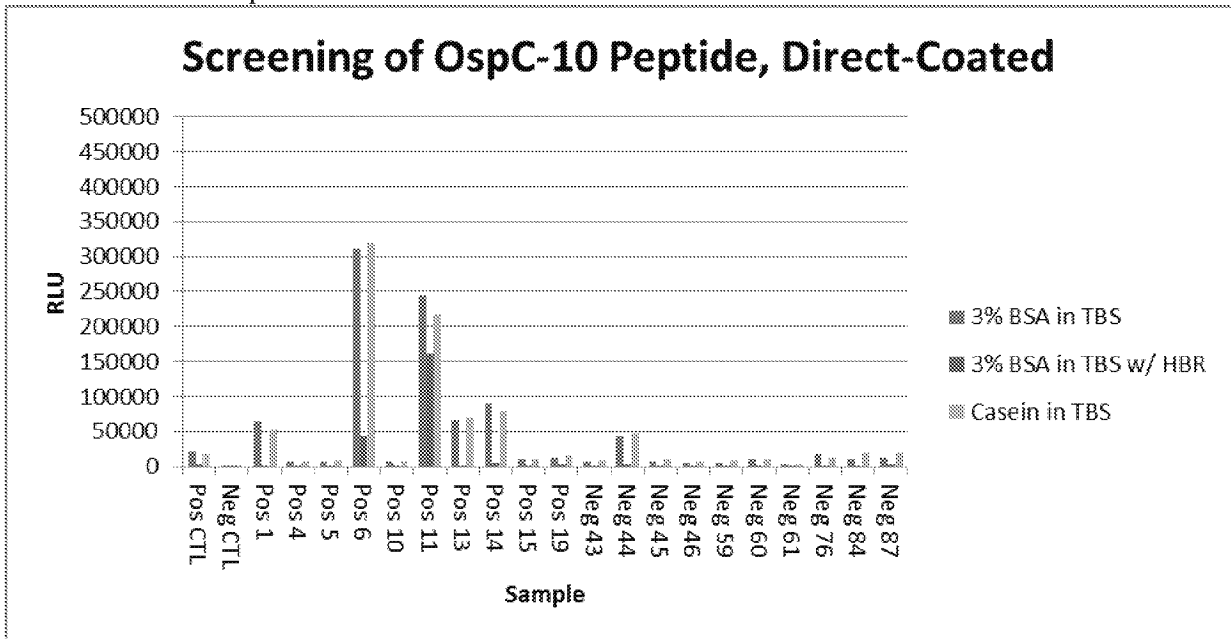
**Figure [ SEQ Figure \\* ARABIC ]:** Screening of C6-25 Biotinylated Peptide with 3 Sample Diluents



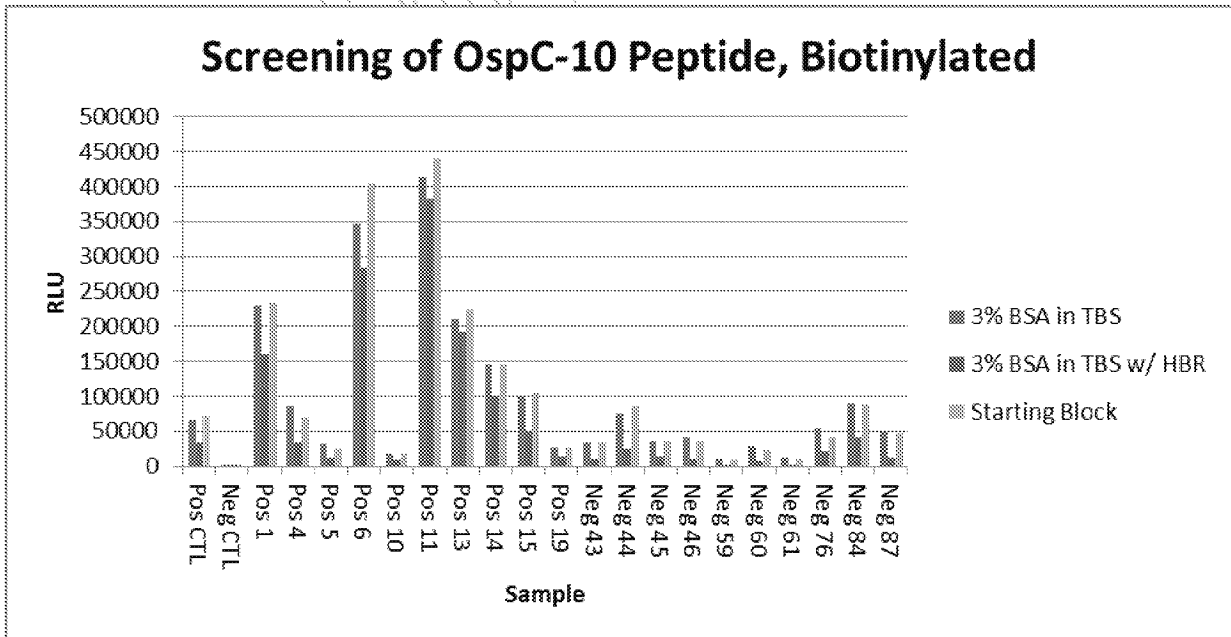
**Table [ SEQ Table \\* ARABIC ]:** Screening of OspC-10 Custom Peptide on a Microtiter Plate

Surface:		OspC-10 Passive Absorption			OspC-10-biotin on Avidin		
Sample Diluent:		3% BSA in TBS	3% BSA in TBS w/ HBR	Casein in TBS	3% BSA in TBS	3% BSA in TBS w/ HBR	Starting Block
Sample Type	Sample #	RLU	RLU	RLU	RLU	RLU	RLU
Pos CTL		21618	4565	18822	65784	34658	72155
Neg CTL		1042	309	620	1303	474	1189
Modulation +/-		21	15	30	50	73	61
Positive Samples	1	65670	2883	52620	229411	160437	233783
	4	7810	1847	7308	85794	34247	70725
	5	7727	1501	8428	32165	13378	25063
	6	310846	43301	319263	347083	284813	403828
	10	8095	2324	7070	18967	9199	17440
	11	243761	161280	216757	413027	382313	439519
	13	66073	2832	69545	211018	192679	224511
	14	90133	5681	78797	145222	101111	146184
	15	11270	2544	11479	100621	50153	105017
	19	12134	3803	15519	26951	14474	26337
Pos Mean RLU		82352	22800	78678	161026	124280	169241
Negative Samples	br43	7168	1792	9029	34598	10185	33660
	br44	43514	3121	46511	75151	24590	85788
	br45	7363	1380	11299	35789	14916	36658
	br46	6256	2381	7056	40853	10250	35544
	br59	4962	1652	8562	10463	2627	9179
	br60	10207	1839	10277	28648	8316	24323
	br61	3208	735	3537	13157	2846	11379
	br76	17820	1753	13164	53871	21017	41112
	br84	10445	1459	19885	89505	40671	88154
	br87	13091	3749	20084	51184	12701	46570
Neg Mean RLU		12403	1986	14941	43322	14812	41237
Modulation Mean Pos/Neg Samples		7	11	5	4	8	4

**Figure [ SEQ Figure \\* ARABIC ]:** Screening of OspC-10 Peptide Direct-Coated with 3 Sample Diluents



**Figure [ SEQ Figure \\* ARABIC ]:** Screening of OspC-10 Biotinylated Peptide with 3 Sample Diluents





## 1.4 Surface Titration

The C6-25 peptide was titrated with both direct absorption and biotin-avidin coating methods. The samples were diluted 1:25 into 3% BSA in TBS Blocking Buffer with and without 400 ug/mL HBR. The positive and negative pools consisted of equal volumes of the 10 consensus positive and negative samples used for screening in previous steps.

With the biotinylated C6 peptide signal was much higher on the Theranos System and it was possible to titrate the concentration of the peptide down to 0.5 ug/mL to achieve maximum differentiation between positive and negative pooled samples.

Under all surface coating conditions, with HBR blocker in the diluent the background signal from negative samples was greatly reduced while the signal from positive samples was not changed.

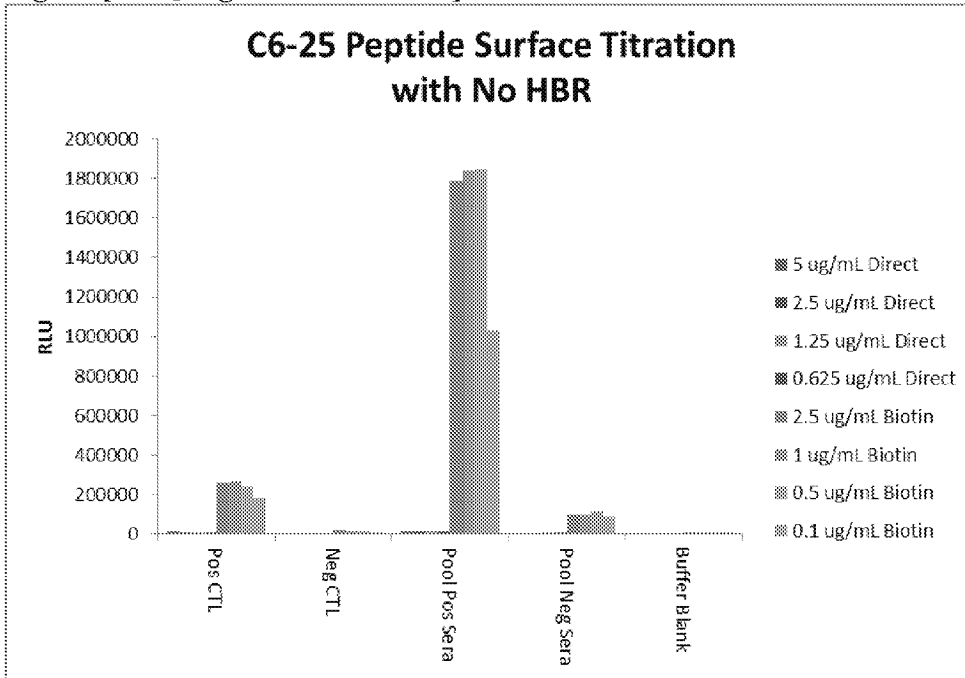
**Table [ SEQ Table \\* ARABIC ]:** Titration of Direct-Coated C6-25 Peptide on the Theranos System

Diluent	Sample	5 ug/mL		2.5 ug/mL		1.25 ug/mL		0.625 ug/mL	
		Mean RLU	CV %	Mean RLU	CV %	Mean RLU	CV %	Mean RLU	CV %
3% BSA TBS	Pos CTL	14446	19.2	12301	11.3	9411	19.5	10696	35.2
	Neg CTL	465	27.9	410	38.7	266	13.7	412	11.3
	Pool Pos Sera	16585	5.0	17551	1.1	17116	19.7	13930	8.8
	Pool Neg Sera	7324	21.0	7244	8.2	8093	22.1	7822	27.8
	Buffer Blank	428	16.0	351	11.4	321	11.2	404	7.8
	<b>Pos/Neg CTL</b>	<b>31</b>		<b>30</b>		<b>35</b>		<b>26</b>	
	<b>Pos/Neg Pooled Sera</b>	<b>2</b>		<b>2</b>		<b>2</b>		<b>2</b>	
3% BSA TBS with HBR	Pos CTL	11442	25.0	8601	14.7	8903	5.6	8248	31.4
	Neg CTL	988	28.1	853	18.4	960	43.1	930	11.0
	Pool Pos Sera	12262	10.7	12621	11.4	11610	5.8	15627	44.2
	Pool Neg Sera	1599	8.3	1735	10.5	1882	7.9	1506	27.2
	Buffer Blank	928	8.7	877	9.3	958	3.4	1021	8.8
	<b>Pos/Neg CTL</b>	<b>12</b>		<b>13</b>		<b>9</b>		<b>9</b>	
	<b>Pos/Neg Pooled Sera</b>	<b>8</b>		<b>7</b>		<b>6</b>		<b>10</b>	

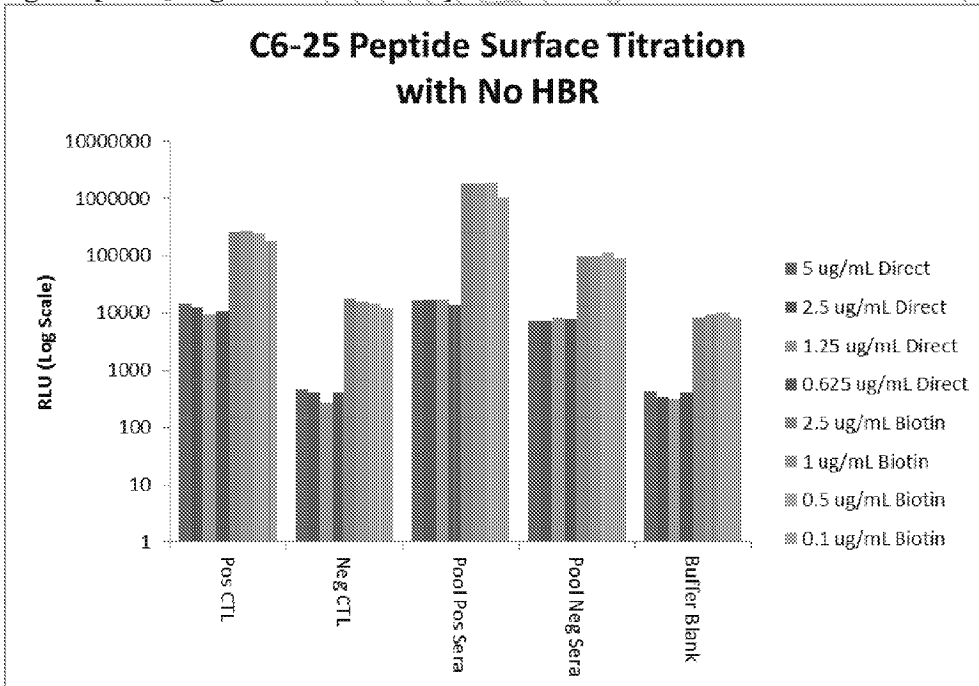
**Table [ SEQ Table \\* ARABIC ]:** Titration of C6-25 Biotin Peptide on Avidin Surface on the Theranos System

Diluent	Sample	2.5 ug/mL		1 ug/mL		0.5 ug/mL		0.1 ug/mL	
		Mean RLU	CV %	Mean RLU	CV %	Mean RLU	CV %	Mean RLU	CV %
3% BSA TBS	Pos CTL	258282	0.7	267859	3.6	237900	17.4	181308	21.5
	Neg CTL	18080	25.4	15624	16.4	14759	22.6	11691	29.7
	Pool Pos Sera	1786335	7.8	1837104	8.8	1846494	8.8	1031795	23.2
	Pool Neg Sera	98580	37.0	95558	38.6	112839	27.1	88642	22.7
	Buffer Blank	8373	34.9	9188	25.1	9962	25.0	8297	27.6
	<b>Pos/Neg CTL</b>	<b>14</b>	<b>17</b>	<b>16</b>	<b>16</b>				
<b>Pos/Neg Pooled Sera</b>	<b>18</b>	<b>19</b>	<b>16</b>	<b>12</b>					
3% BSA TBS with HBR	Pos CTL	130413	10.9	161948	3.0	145160	13.2	127553	21.0
	Neg CTL	13325	13.8	13216	14.8	11637	14.9	11812	21.4
	Pool Pos Sera	1447703	11.4	1463572	15.2	1551160	9.6	569815	12.1
	Pool Neg Sera	21678	15.9	19393	9.8	20752	18.2	18341	2.8
	Buffer Blank	11192	30.4	11821	17.7	11556	2.5	11158	25.7
	<b>Pos/Neg CTL</b>	<b>10</b>	<b>12</b>	<b>12</b>	<b>11</b>				
<b>Pos/Neg Pooled Sera</b>	<b>67</b>	<b>75</b>	<b>75</b>	<b>31</b>					

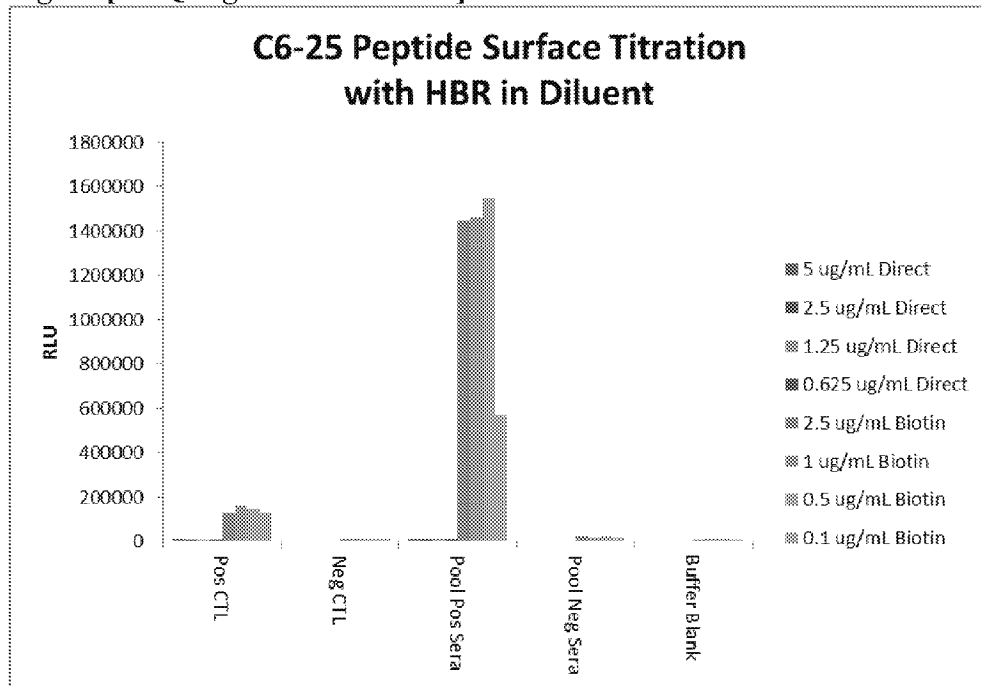
**Figure [ SEQ Figure \\* ARABIC ]: C6-25 Surface Titration without HBR**



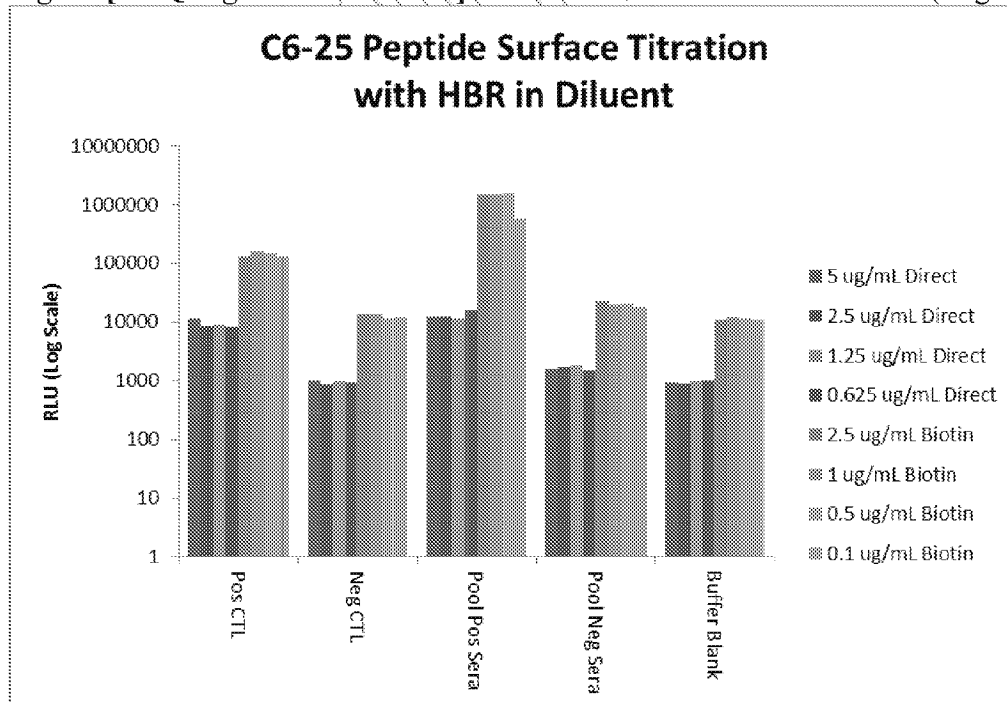
**Figure [ SEQ Figure \\* ARABIC ]: C6-25 Surface Titration without HBR (Log Scale)**



**Figure [ SEQ Figure \\* ARABIC ]: C6-25 Surface Titration with HBR**



**Figure [ SEQ Figure \\* ARABIC ]: C6-25 Surface Titration with HBR (Log Scale)**



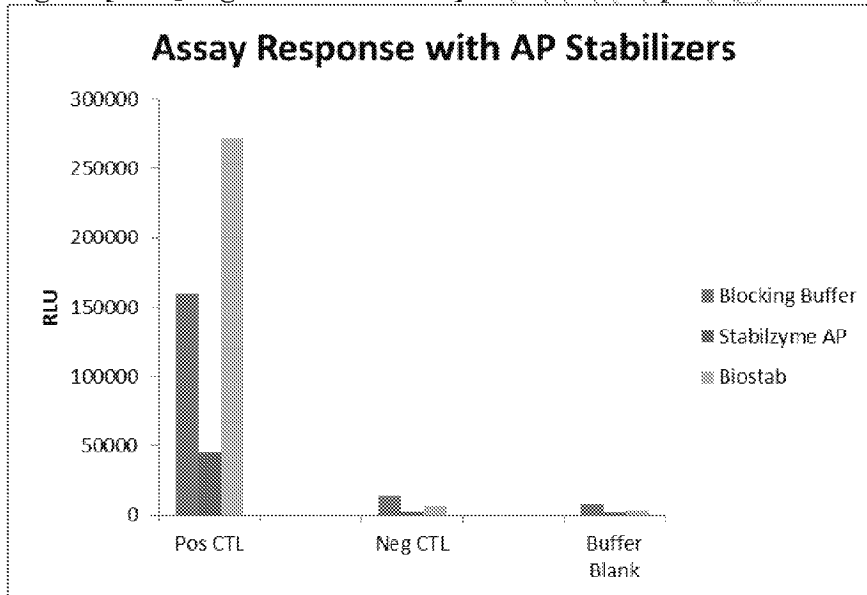
## 1.5 Alkaline Phosphatase Stabilizers

Two commercial alkaline phosphatase stabilizers were tested as detection antibody diluents, with the IgG DAb at 100 ng/mL and IgM DAb at 25 ng/mL. The samples were diluted 1:25 into 3% BSA in TBS Blocking Buffer with 400ug/mL HBR. Signal modulation as best with Biostab.

**Table [ SEQ Table \\* ARABIC ]:** Alkaline Phosphatase Stabilizers

Sample	Blocking Buffer		Stabilzyme AP		Biostab	
	Mean RLU	CV %	Mean RLU	CV %	Mean RLU	CV %
Pos CTL	160016	15.6	45439	15.5	271915	12.3
Neg CTL	14202	5.0	2492	12.7	6295	8.5
Buffer Blank	7955	13.6	1751	19.2	3477	28.0
Pos/Neg CTL	11		18		43	
Pos/Background	20		26		78	

**Figure [ SEQ Figure \\* ARABIC ]:** Alkaline Phosphatase Stabilizers



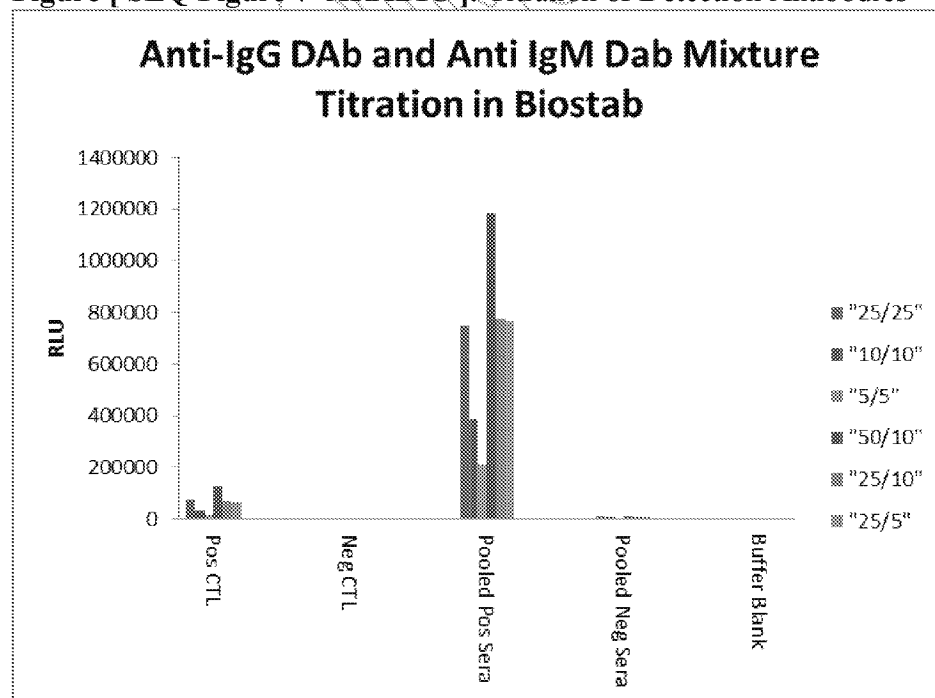
## 1.6 Detection Antibody Titration

The AP conjugated detection antibodies were titrated in Biostab. The best modulation between the positive and negative control and the pooled positive and negative sera was achieved with 25 ng/mL of the anti-IgG DAb and 5 ng/mL of the anti-IgM DAB.

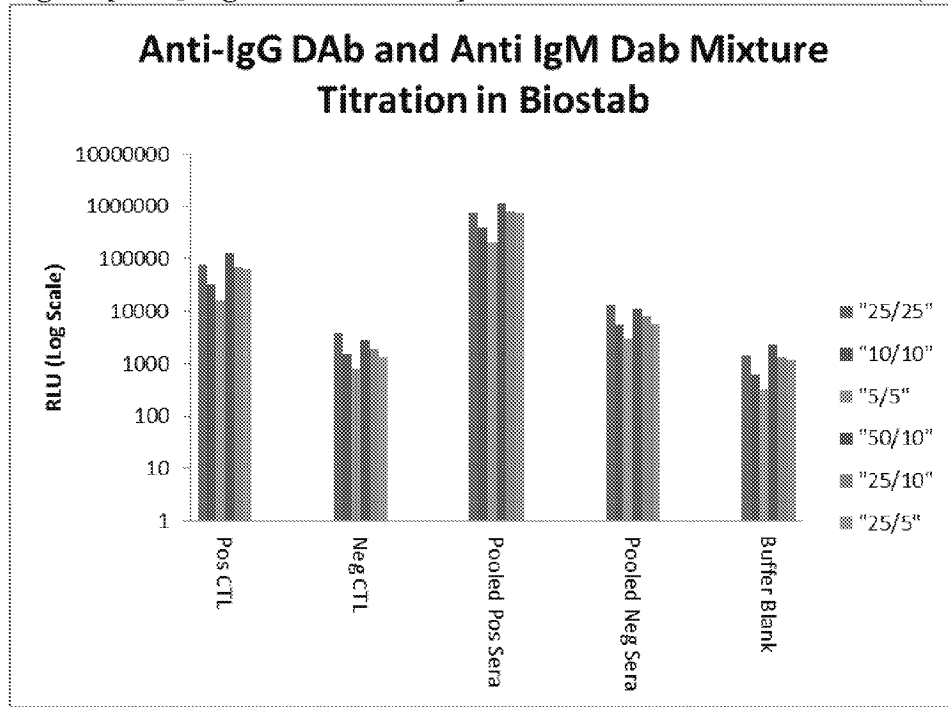
**Table [ SEQ Table \\* ARABIC ]:** Titration of Detection Antibodies

[IgG DAb]:	25 ng/mL	10 ng/mL	5 ng/mL	50 ng/mL	25 ng/mL	25 ng/mL
[IgM DAb]:	25 ng/mL	10 ng/mL	5 ng/mL	10 ng/mL	10 ng/mL	5 ng/mL
Sample	Mean RLU	Mean RLU	Mean RLU	Mean RLU	Mean RLU	Mean RLU
Pos CTL	76272	32194	16480	125660	70585	65377
Neg CTL	3796	1496	795	2816	1963	1316
Pooled Pos Sera	745573	388324	211221	1182513	778715	763042
Pooled Neg Sera	13195	5399	3003	11282	8021	5825
Buffer Blank	1436	636	326	2378	1321	1164
Kit Pos/Neg CTL	20.1	21.5	20.7	44.6	36.0	49.7
Pooled Pos/Neg Sera	57	72	70	105	97	131
Pos Sera/Background	519	611	648	497	589	656

**Figure [ SEQ Figure \\* ARABIC ]:** Titration of Detection Antibodies



**Figure [ SEQ Figure \\* ARABIC ]:** Titration of Detection Antibodies (Log Scale)



### 1.7 Reagent Incubation Time

The effect of shorter reagent incubation times was tested with sample, detection conjugate and substrate incubation times respectively of 10, 10, 10; 5, 5, 5; and 2, 2, 1 minutes. Assay modulation was similar with 5 minute and 10 minute incubations, but with 2, 2, 1 minute incubations the modulation fell off sharply. The 5,5,5 minute incubation protocol was chosen as the final condition.

**Table [ SEQ Table \\* ARABIC ]:** Reagent Incubation Time

Incubation Time (Minutes):	10, 10, 10	5, 5, 5	2, 2, 1
Sample	Mean RLU	Mean RLU	Mean RLU
Pos CTL	65377	19787	2304
Neg CTL	1316	610	173
Pooled Pos Sera	763042	210967	35544
Pooled Neg Sera	5825	1734	280
Buffer Blank	1164	363	195
<b>Kit Pos/Neg CTL</b>	<b>49.7</b>	<b>32.5</b>	<b>13.3</b>
<b>Pooled Pos/Neg Sera</b>	<b>131</b>	<b>122</b>	<b>127</b>
<b>Pos Sera/Background</b>	<b>656</b>	<b>581</b>	<b>182</b>

Figure [ SEQ Figure \\* ARABIC ]: Reagent Incubation Times

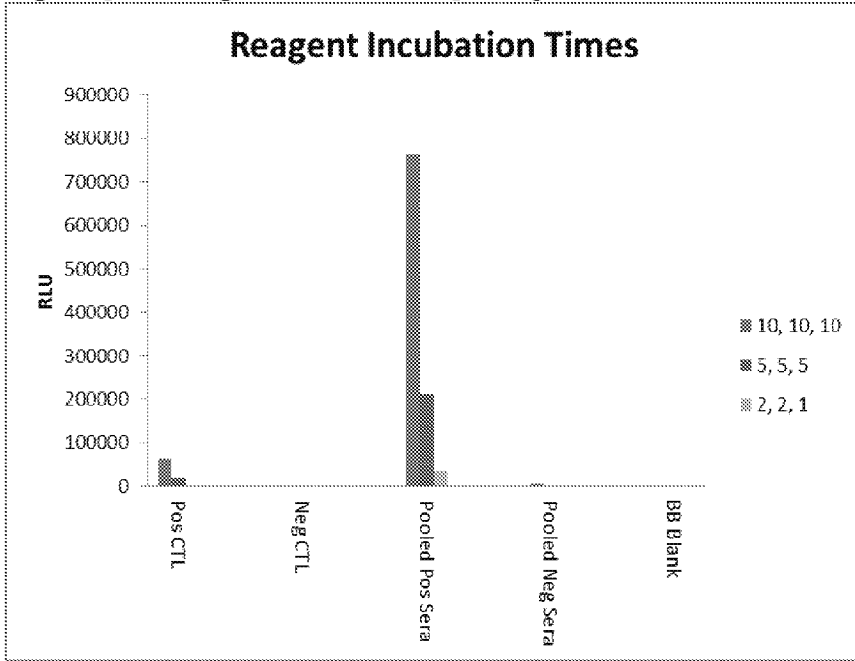
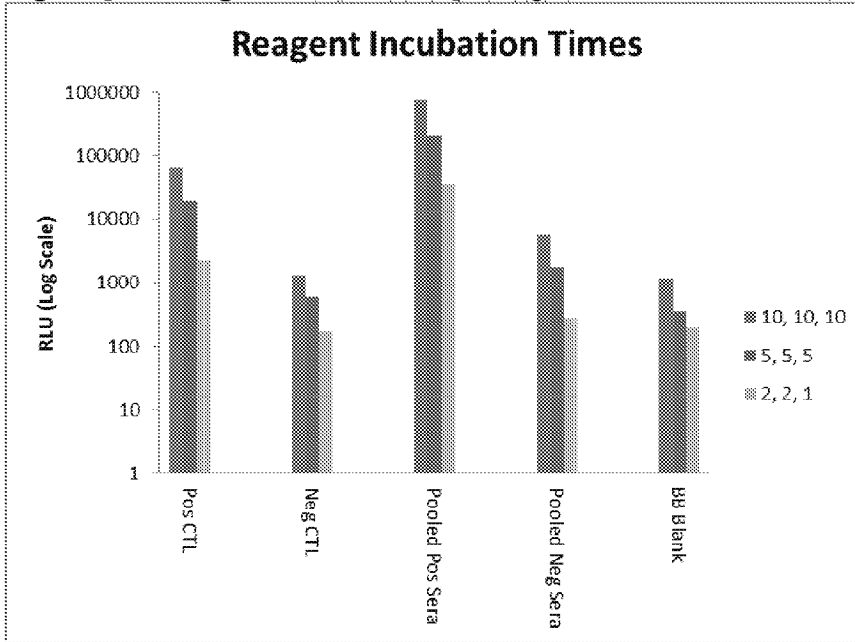


Figure [ SEQ Figure \\* ARABIC ]: Reagent Incubation Times (Log Scale)





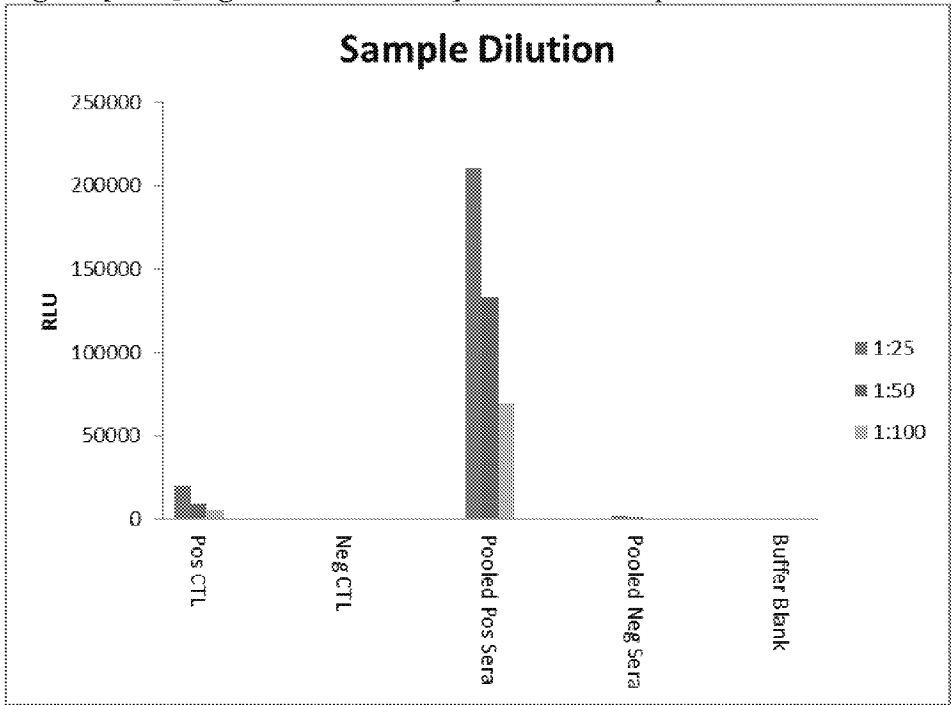
## 1.8 Sample Dilution

The effect of sample dilution was tested with final sample dilution factors of 1:25, 1:50 and 1:100 into 3% BSA in TBS blocking buffer with 400ug/mL HBR. Modulation between pooled positive and negative sera was best at 1:50 as a result of a greater reduction in the signal from negative samples compared to the reduction in signal from the positive samples.

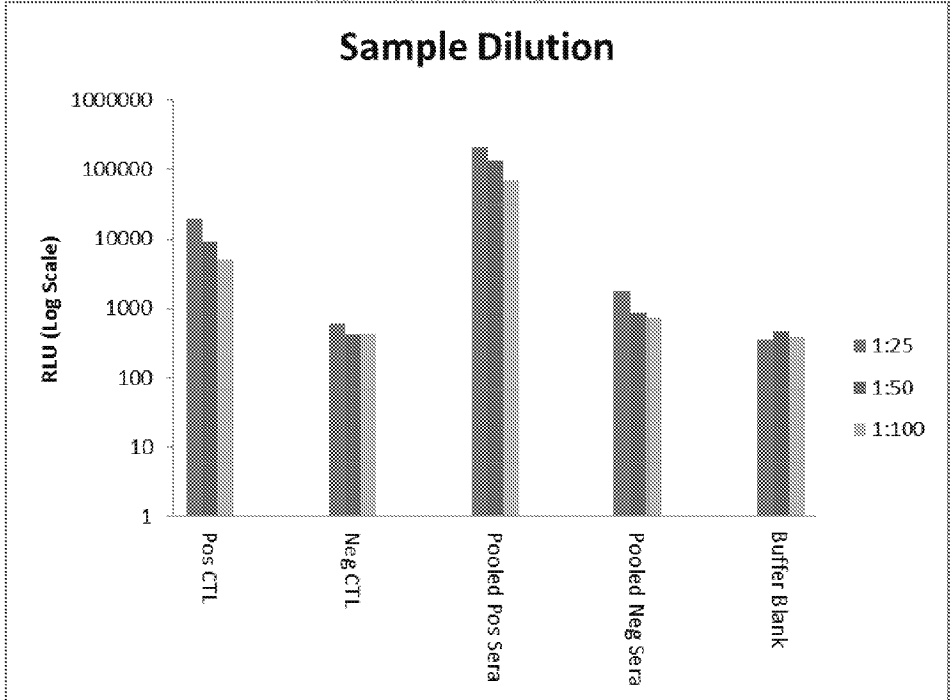
**Table [ SEQ Table \\* ARABIC ]:** Effect of Sample Dilution

Sample Dilution:	1:25	1:50	1:100
Sample	Mean RLU	Mean RLU	Mean RLU
Pos CTL	19787	9051	5019
Neg CTL	610	408	424
Pooled Pos Sera	210967	133274	69420
Pooled Neg Sera	1734	877	719
Buffer Blank	363	459	381
Kit Pos/Neg CTL	32.5	22.2	11.8
Pooled Pos/Neg Sera	122	152	97

**Figure [ SEQ Figure \\* ARABIC ]:** Effect of Sample Dilution



**Figure [ SEQ Figure \\* ARABIC ]:** Effect of Sample Dilution (Log Scale)



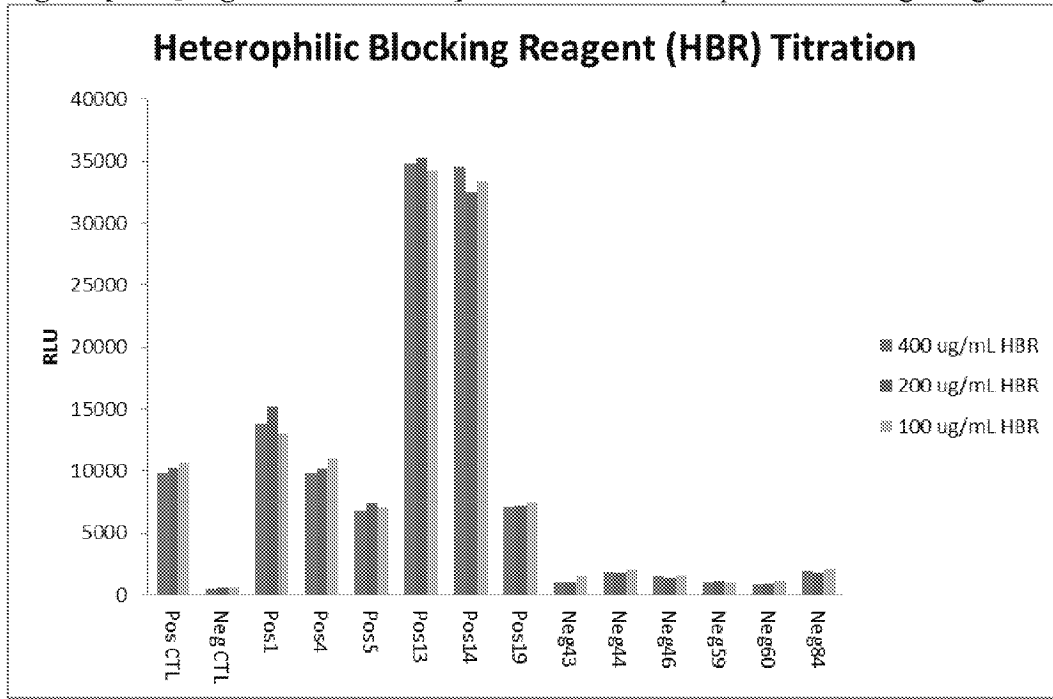
## 1.9 Titration of Heterophilic Blocking Reagent

The concentration of HBR in the 3% BSA in TBS blocking buffer sample diluent was titrated using 6 samples tested as positive and low-positive in the 3 assays, and 6 samples tested as high-negative in the 3 assays. At 200ug/mL the mean signal from negative samples was similar to that with 400ug/mL but when the HBR concentration was reduced to 100ug/mL, the mean signal for negative samples increased. To maintain the sensitivity of the assay, 200ug/mL HBR was chosen as the final concentration.

**Table [ SEQ Table \\* ARABIC ]:** Titration of Heterophilic Blocking Reagent

[HBR], ug/mL:		400	200	100
Sample		Mean RLU	Mean RLU	Mean RLU
Pos CTL		9839	10201	10633
Neg CTL		499	553	538
Modulation Pos/Neg CTL		20	18	20
ProMedDx Positive	1	13848	15215	13060
	4	9802	10182	11042
	5	6790	7329	7017
	13	34794	35288	34210
	14	34543	32467	33372
	19	7070	7159	7482
Mean RLU Pos Samples		17808	17940	17697
Bioreclamation Normal	br43	1000	969	1517
	br44	1873	1827	2040
	br46	1512	1342	1624
	br59	962	1072	982
	br60	795	906	1070
	br84	1991	1780	2140
Mean RLU Neg Samples		1356	1316	1562
Modulation Mean (Pos)/Mean(Neg)		13	14	11

**Figure [ SEQ Figure \\* ARABIC ]:** Titration of Heterophilic Blocking Reagent



Theranos

## 1.10 Clinical Samples and Cutoff Determination

Lyme disease antibody positive sera (30) and normal sera (30) were obtained and tested in the commercial ELISA kits and in the Theranos System.

The Theranos cutoff value was determined by taking the mean RLU of the normal samples plus 10 times the standard deviation of the 30 normal samples. The sample RLU divided by the cutoff value yields the Antibody Index.

There were no false positives in the Immunitics or Theranos assay (100% agreement) in the 30 normal samples, whereas in the US Biological assay 3 of the normal sera showed positive results and one showed an equivocal result.

The samples obtained as Lyme antibody positive from ProMedDx were determined positive in the VIDAS system at the time of collection, but no diagnosis data was available. Of these 30 samples, 8 were negative in all 3 tests, while 6 more were negative or equivocal in the Immunitics and Theranos assay while positive in the US Biological assay.

Excepting sample #10919970 which was equivocal in the Theranos assay and positive in the US Biological assay, there was 83% agreement between Theranos and US Biological for this sample set.

There was 100% agreement between Immunitics and Theranos in this sample set except sample #10919970 was equivocal in the Theranos assay while negative in the Immunitics assay representing a borderline agreement.

**Table [ SEQ Table \\* ARABIC ]: Results for 30 Normal Sera**

Sample	US Biological Ab Index	Immunetics Ab Index	Theranos Ab Index
BRH351243	0.44	0.19	0.15
BRH351244	0.54	0.47	0.28
BRH351245	0.34	0.24	0.12
BRH351246	0.52	0.30	0.21
BRH351257	1.30	0.20	0.11
BRH351259	0.60	0.16	0.17
BRH351260	0.47	0.24	0.14
BRH351261	0.75	0.27	0.14
BRH351262	0.83	0.34	0.24
BRH351264	1.04	0.17	0.17
BRH351265	0.43	0.11	0.17
BRH351267	0.36	0.16	0.11
BRH351268	0.50	0.11	0.14
BRH351269	0.56	0.29	0.21
BRH351272	0.59	0.20	0.22
BRH351276	0.74	0.34	0.47
BRH351277	1.12	0.17	0.21
BRH351279	0.60	0.16	0.12
BRH351280	0.54	0.30	0.12
BRH351281	0.37	0.16	0.20
BRH351282	0.71	0.21	0.36
BRH351284	0.61	0.17	0.27
BRH351285	0.60	0.15	0.10
BRH351286	1.24	0.16	0.13
BRH351287	0.53	0.16	0.14
BRH351288	0.61	0.16	0.23
BRH351288	0.76	0.12	0.11
BRH351289	0.48	0.13	0.13
BRH351290	0.68	0.18	0.20
BRH351291	0.51	0.27	0.19

Ab Index > 1.1
Ab Index > 0.9, < 1.1
Ab Index < 0.9

**Table [ SEQ Table \\* ARABIC ]: Results for 30 Lyme Disease Antibody Positive Sera\***

Sample	US Biological Ab Index	Immunetics Ab Index	Theranos Ab Index
10915791	3.49	4.43	2.35
10919479	0.54	0.25	0.32
10919480	1.17	0.43	0.53
10919776	3.96	4.71	1.57
10919778	1.66	2.09	1.13
10919847	5.15	9.18	113.95
10919848	0.58	0.26	0.24
10919849	0.61	0.22	0.21
10919906	2.06	0.58	0.33
10919907	2.72	8.36	24.21
10919908	3.79	9.06	14.76
10919970	3.59	0.23	1.01
10919972	3.33	4.71	5.45
10919973	2.14	7.00	5.01
10920987	4.33	9.10	36.45
10921154	1.17	0.24	0.86
10921214	0.89	0.33	0.25
10921215	2.31	0.55	0.50
10921305	2.03	2.81	1.26
10921353	0.83	0.29	0.38
10924272	4.29	4.96	4.53
10924273	0.83	0.20	0.26
10924274	3.21	0.20	0.45
10924275	0.47	0.14	0.14
10924276	1.81	2.65	1.71
10924347	3.54	7.24	6.07
10924348	0.76	0.17	0.24
10924351	3.38	6.55	6.40
10924352	4.07	7.74	17.42
10924353	1.84	2.12	1.21

Ab Index > 1.1
Ab index > 0.9, < 1.1
Ab Index < 0.9

\* Sera determined positive by VIDAS, not by diagnosis.

## 1.11 Specificity

Samples positive for conditions known to cause false positives in Lyme Disease antibody assays were tested on the Theranos assay to test for specificity.

All rheumatoid factor (RF) positive and anti-cardiolipin positive samples were negative in all 3 assays. The Human Anti-Mouse Antibodies (HAMA) positive samples were all negative in the Theranos assay and not tested in the commercial assays.

Of 14 anti-nuclear antibody (ANA) positive samples, one was positive in the Theranos assay and negative in the other 2 assays. One sample was strongly positive in all 3 assays. It should be assumed that samples positive for ANA could cause false positives in Lyme Disease antibody assays and that patients with ANA may concurrently have Lyme disease so sample #31 may not be considered a false positive unless verified by western blot to be negative for Lyme antibodies.

The Immunetics ELISA also documents cross reactivity with ANA (1 in 18 samples tested), RA (1 in 16 samples tested), and Syphilis (1 in 20 samples tested) according to the kit insert.

For assay validation, samples positive for syphilis and HIV should also be assayed in a suitable facility to screen for cross reactivity with these conditions.



**Table [ SEQ Table \\* ARABIC ]: Specificity**

Condition	Sample #	Result	US Biological Ab Index	Immunitics Ab Index	Theranos Ab Index
Rheumatoid Factor (RF) Positive	SFB7885		0.49	0.17	0.18
	SFB7886		0.49	0.13	0.30
	SFB7887		0.46	0.20	0.23
	SFB7888		0.35	0.13	0.26
	SFB78893		0.56	0.19	0.32
	SFB7891		0.58	0.16	0.15
IIAMA Positive (Result in ng/mL)	10580298	48.0	n/a	n/a	0.55
	10798282	68.1	n/a	n/a	0.19
	10840150	54.9	n/a	n/a	0.72
	10867314	45.4	n/a	n/a	0.45
	10958926	40.0	n/a	n/a	0.49
	10958975	41.2	n/a	n/a	0.39
Anti-Cardiolipin Positive	BRH353851*		0.41	0.10	0.27
	BioRad Pos CTL		n/a	n/a	0.15
	BioRad Weak Pos CTL		n/a	n/a	0.09
	ASI Pos CTL		n/a	n/a	0.17
Anti-Nuclear factor (ANA) Positive (Titer)	ANAPos9	1:1280	0.72	0.21	1.31
	ANAPos11	1:1280	0.46	0.20	0.23
	ANAPos17	1:1280	0.59	0.10	0.50
	ANAPos18	1:640	0.57	0.09	0.25
	ANAPos19	1:640	0.88	0.15	0.51
	ANAPos20	1:640	0.65	0.25	0.26
	ANAPos24	1:1280	0.67	0.13	0.33
	ANAPos25	1:640	0.59	0.16	0.24
	ANAPos26	1:640	0.47	0.13	0.21
	ANAPos27	1:640	0.50	0.69	0.21
	ANAPos28	1:640	0.58	0.15	0.55
	ANAPos29	1:160	0.51	0.14	0.22
	ANAPos30	1:160	0.52	0.21	0.37
	ANAPos31	1:320	2.91	13.73	28.47

\* Sample determined positive in Cardiolipin ELISA Kit #BQ0771

Ab Index > 1.1
Ab Index > 0.9, < 1.1
Ab Index < 0.9

## 1.12 Stability

Stability monitoring is ongoing for the the assay reagents stored at 4°C and protected from light.

**Table [ SEQ Table \\* ARABIC ]: Stability of Reagents**

Date	Day	Sample	Signal (RLU)		Calculated Antibody Index	
			Mean RLU	CV %	Mean Ab Index	CV %
9/16/11	0	Pos CTL	65797	2.3	10.4	3.1
		Neg CTL	1307	6.1	0.2	5.0
10/27/11	41	Pos CTL	65198	10.1	9.9	6.8
		Neg CTL	1391	8.3	0.2	10.2

Figure [ SEQ Figure \\* ARABIC ]: Stability, Signal (RLU)

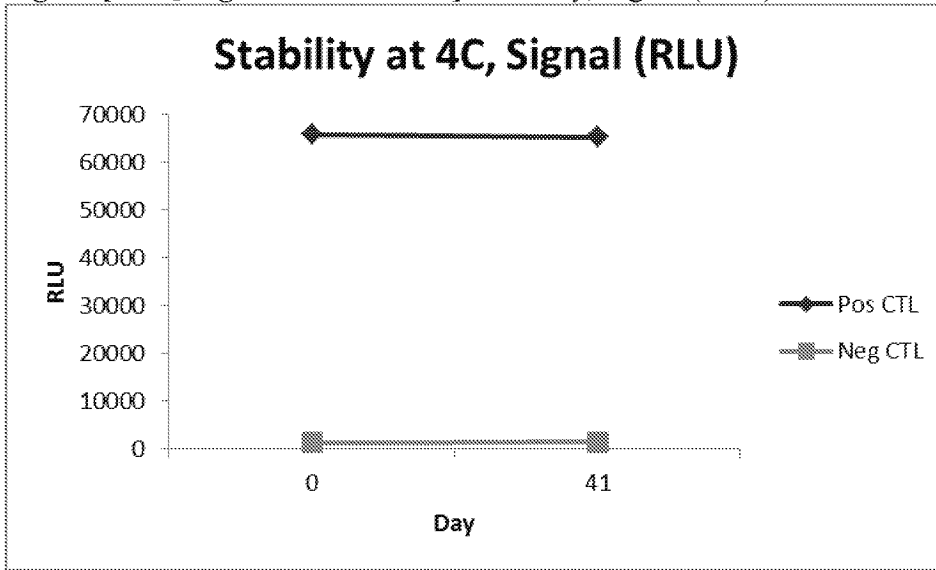
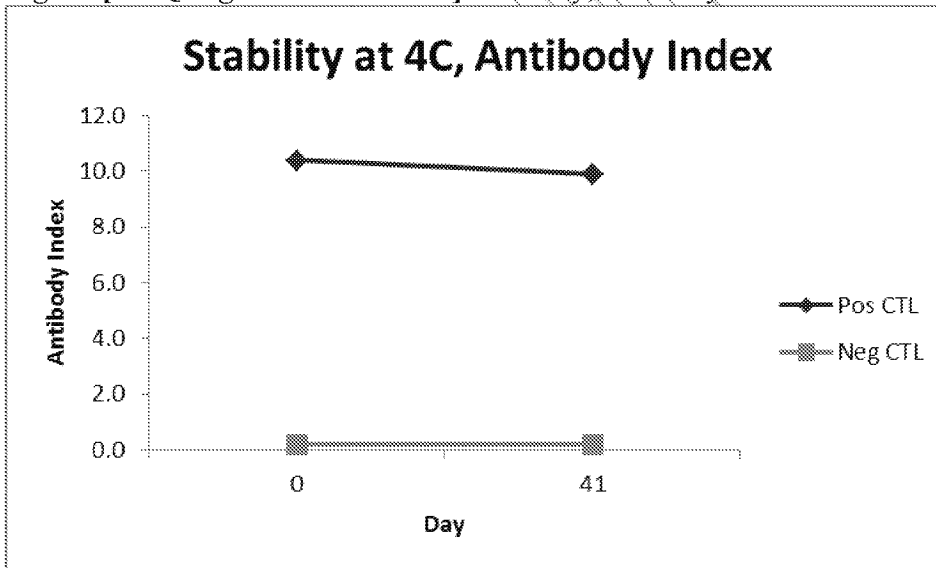


Figure [ SEQ Figure \\* ARABIC ]: Stability, Antibody Index



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