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Epstein-Barr Virus IgG 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 semi-quantitatively determine the presence of IgG antibodies to Epstein-Barr virus 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 kit was used in house as a predicate method:

- Diasorin Kit-ETI-VCA-G- Prod #:P001606A

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

A 58 amino acid synthetic peptide serves as the capture surface for Epstein Barr Virus IgG antibodies in the sample. After incubation of the appropriately-diluted sample on the capture surface, the surface is washed. An alkaline phosphatase-labeled mouse anti-human IgG detection antibody is incubated on the surface. After this incubation period, the surface is washed again. 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

Name	Supplier	Catalog #
Carbonate-bicarbonate buffer	Sigma	C3041
Heterophilic Blocking Reagent (HBR)	Scantibodies	3KC533
Starting Block	Thermo Fisher	37542
Assay Diluent	Surmodics	Prod #:SM 01-1000
Phospho Glo Substrate	KPL	55-60-04

Table [SEQ Table * ARABIC]: Antigens

Antigen #	Vendor	Product Code	Description
1	USBiological	E3440-27	Viral Capsid Antigen p23, aa1-162, Recombinant
2	USBiological	E3440-26	Viral Capsid Antigen p18, aa1-119, Recombinant
3	Microbix Biosystems	EL-16-06-001	VCA-Immunoaffinity purified gp125 protein
4	GenWay Biotech, Inc.	GWB-A7992B	Epstein Barr Virus (EBV-VCA) protein
5	Biosyn	EBV_Ori_p18_58aa (biotinylated)	BIOTIN-STAVAQSATPSVSSSISSLRAATSGATAAAS AAAAVDTGSGGGGQPHDTAPRGARKKQ
6	Biosyn	EBV_Ori_p18_58aa	STAVAQSATPSVSSSISSLRAATSGATAAAS AAAAVDTGSGGGGQPHDTAPRGARKKQ
7	Biosyn	EBV_Alt_p18_56aa (biotinylatcd)	BIOTIN-STAVAQSATPSVSSSISSLRAATSGATAAA CCAVDTGSGGGGQPHDTAPRGARKKQ
8	Biosyn	EBV_Alt_p18_56aa	STAVAQSATPSVSSSISSLRAATSGATAAA CCAVDTGSGGGGQPHDTAPRGARKKQ

Table [SEQ Table * ARABIC]: Detection Antibodies

DAb #	Supplier	Catalog #	Description
1	Novus	NB100-2046	Mouse Anti-Human IgG Antibody
2	US Biological	I904-75W	Mouse Anti-Human IgG Antibody

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

2.1 Capture Surface: Antigen Screen (MTP)

Clinical samples were screened on the Diasorin Kit to determine positive and negative EBV IgG samples. Commercially available antigens were then screened with these positive and negative samples on a microtiter plate (MTP). Each antigen was prepared in carbonate bi carbonate buffer for direct coating on the Nunc-384 well plates. With the exception of Antigen 3 coated at 3 ug/mL, all antigens were coated at 10 ug/ml. A sample dilution of 1:25 was added to the surface followed by wash steps. The detection antibody (Mouse Anti-Human IgG from Novus) concentration was 100 ng/ml in 3% BSA in TBS blocking buffer. Antigens #5,#6,#7, and #8 showed good modulation between positive and negative samples. Both direct coating of the raw antigen by passive absorption and ultraavidin coating of the biotinylated antigen were tested on the MTP for Antigen 5.

Table [SEQ Table * ARABIC]: Capture Surface Screen (MTP)

Antigen #	**1**		**2**		**3**		**4**	
[Antigen], ug/ml	**10ug/ml**		**10ug/ml**		**3 ug/ml**		**10ug/ml**	
Sample#	**Mean Value**	**CV%**	**Mean Value**	**CV%**	**Mean Value**	**CV%**	**Mean Value**	**CV%**
Negative: #1	160897	4.4	971	7.9	2876	15.5	1119	16.1
#2	274202	4.9	3284	7.6	2966	7.5	1312	4.5
Mean Negative	**217550**		**2128**		**2921**		**1216**	
Positive: #3	85389	4.4	1478	5.7	58640	5.3	651	8.1
#4	106796	2.4	2174	8.1	31654	13.1	1188	8.9
#5	119701	5.1	1862	9.9	28105	2.9	1427	13.1
#6	93543	2.2	4530	10.3	148307	7.1	1275	7.6
#7	137966	3.3	13233	17.3	217401	7.7	1278	6.6
#8	170802	4.4	12058	8.8	489788	3.2	2351	9.5
#9	213086	2.9	3576	4.8	84130	1.6	3395	8.5
Mean Positive	**132469**		**5559**		**151146**		**1652**	
Modulation	**0.6**		**2.6**		**52**		**1.4**	
No Sample*	43192	9.3	353	11.7	3853	1.6	513	11.5

*No Sample: Blocking buffer blank with detection antibody and substrate.

Table 3- continued

Antigen #	5		6		7		8	
[Antigen], ug/ml	10ug/ml		10ug/ml		10ug/ml		10ug/ml	
Sample#	Mean Value	CV%	Mean Value	CV%	Mean Value	CV%	Mean Value	CV%
Negative: #1	1801	10	1445	6.8	3520	8.9	4302	20.6
#2	2827	2.8	2464	2	5276	2.3	4816	3.1
Mean Negative	**2314**		**1954**		**4398**		**4559**	
Positive: #3	69082	2.9	66190	4.2	81184	1.4	74753	2.2
#4	463475	1.9	438129	7.7	511376	0.8	482382	3
#5	22136	3.1	22691	3.7	26739	8.7	24398	5.3
#6	160327	1.4	159402	2.6	189968	2	173757	6.2
#7	1004560	2	1085271	2.6	1102049	7.2	1084435	4.7
#8	1171215	0.7	1215861	5.4	1334521	1.6	1283435	3.3
#9	17931	8.3	27243	12.7	19922	1.5	23565	21.7
Mean Positive	**415532**		**430684**		**466537**		**449532**	
Modulation	**180**		**220**		**106**		**99**	
No SAMPLE*	2643	9.8	2863	1.5	1450	0.2	1407	9.7

*No Sample: Blocking buffer blank with detection antibody and substrate.

Table 4 [SEQ Table * ARABIC]: Antigen Coating Methods (MTP)

	Avidin Surface		Direct Coat	
Sample ID	**MeanValue**	**CV%**	**MeanValue**	**CV%**
Negative: #1	8990	11.5	1801	10
#2	9691	7.9	2827	2.8
Mean Negative	**9340**		**2314**	
Positive: #3	51586	6.5	69082	2.9
#4	116128	9.4	463475	1.9
#5	19048	9.9	22136	3.1
#6	125588	5.5	160327	1.4
#7	758578	9.1	1004560	2
#8	880388	3.2	1171215	0.7
#9	17494	3.2	17931	8.3
Mean Positive	**281259**		**415532**	
Modulation	**30**		**180**	
No Sample*	754	14	2643	9.8

*No Sample: Blocking buffer blank with detection antibody and substrate.

2.2 Capture Surface Detection Antibody Screen on the Theranos System

Antigen #5 and Antigen #6 are essentially the same peptide with the difference being that Antigen #5 is biotinylated at the N terminal to enable coating onto an avidin surface. Both of these antigens were tested simultaneously with the different detection antibodies. These antigens were screened on the Theranos system at 10 ug/ml under different coating conditions. The detection antibody concentration was 50 ng/ml in 3% BSA Blocking buffer. Clinical samples were tested on the Diasorin Kit, and then used as the test set on the Theranos system. A sample dilution of 1:25 is manually done and the protocol was run at the 10,10,10 minutes incubation time.

There was no significant improvement in terms of modulation between the two antigens, so the biotinylated antigen (Antigen #5) was selected for further optimization. Both DAb 1 and DAb 2 worked well. DAb1 gave slightly higher modulation compared to DAb2 and was used further. DAb2 is a possible back-up detection antibody.

Table [SEQ Table * ARABIC]: Capture Surface Screen-Theranos system

A. Antigen 5 (biotinylated)-Coated on Avidin Surface

Sample Type	Sample#	DAb 1		DAb 2		DAb 3	
		Mean RLU	CV%	Mean RLU	CV%	Mean RLU	CV%
Negative	1	12288	11.7	5776	16.7	94703	7.2
	3	10048	21.2	7304	8.4	141024	14.2
	4	12053	8.8	5974	9.0	65660	9.2
	5	7625	12.5	5869	12.6	56667	18.6
	6	4319	56.6	4897	9.6	47793	23.4
	Mean Neg	**9267**		**5964**		**81169**	
Positive	7	26290	22.9	13642	12.8	283587	7.4
	8	275543	8.2	156176	6.5	1331579	21.9
	9	132634	18.9	92974	11.6	773556	21.1
	10	200317	16.4	86917	12.8	890115	3.7
	11	42215	12.3	17334	11.5	344386	6.0
	12	539268	9.9	250149	8.2	1427492	13.9
	13	89755	25.9	45768	14.5	605329	7.7
	14	103530	14.9	50654	19.2	621291	13.5
	15	63982	15.7	35865	12.2	1626707	7.5
	Mean Pos	**163726**		**83276**		**878227**	
	Modulation	**17.7**		**14.0**		**10.8**	

B. Antigen 6 -Directly Coated

Sample Type	Sample#	Novus DAb Mean RLU	Novus DAb CV%	US Biol DAb Mean RLU	US Biol DAb CV%	Southern Biotech DAb Mean RLU	Southern Biotech DAb CV%
Negative	1	490	31.7	277	27.9	4484	18.3
	3	1636	17.5	838	28.4	21003	26.3
	4	2288	5.7	1313	12.5	21629	16.8
	5	385	29.8	291	6.6	2234	11.0
	6	358	30.6	273	16.3	1585	39.8
	Mean Neg	**1031**		**598**		**10187**	
Positive	7	9201	28.1	3858	13.4	159111	8.6
	8	40151	16.3	16489	15.0	301937	15.5
	9	41376	16.4	18954	8.5	312264	4.8
	10	9185	17.0	3515	35.5	106555	6.2
	11	9421	19.5	4620	16.3	384325	18.3
	12	16358	11.0	7499	9.4	149097	9.6
	13	15913	8.6	6187	30.2	141554	12.5
	14	14154	27.0	7079	12.7	157202	14.0
	15	2681	4.5	1544	24.9	131702	24.7
	Mean Pos	**17604**		**7749**		**204861**	
	Modulation	**17.1**		**13.0**		**20.1**	

2.3 Capture Surface Titration on the Theranos System

The capture surface Antigen #5 (biotinylated) was titrated at the following concentrations: 5 ug/ml, 2.5 ug/ml and 1 ug/ml on an avidin surface. A manual sample dilution of 1:25 was used and the protocol used a 10, 10, 10 minute incubation time. The detection antibody was 50 ng/ml in 3% BSA blocking buffer. Similar modulation was observed for all three concentrations and sensitivity was still good at 1 ug/ml , so this concentration was chosen for further optimization. Clinical samples determined as positive and negative in the Diasorin Kit were used as the test set for the Theranos system.

Table [SEQ Table * ARABIC]: Antigen 5- Capture Titration on the Theranos System

Sample Type	Sample #	5ug/ml Mean RLU	CV%	2.5ug/ml Mean RLU	CV%	1ug/ml Mean RLU	CV%
Negative Samples	1	8957	12.0	9181	12.3	9392	8.4
	3	11193	11.5	11320	10.9	10983	8.4
	4	9264	3.7	8864	23.8	10117	12.5
	5	7219	32.2	7191	25.2	9150	18.2
	6	5690	22.9	4628	17.6	5781	17.0
	Mean Negative	**8465**		**8237**		**9085**	
Positive Samples	7	30417	28.4	33578	13.0	33436	10.3
	8	398015	10.9	402375	16.0	407144	7.4
	9	178323	11.6	180288	28.3	198969	13.3
	10	181220	12.8	173369	12.7	169356	20.4
	11	37086	32.5	49888	16.9	46015	15.7
	12	566603	10.0	556048	4.2	571875	16.3
	13	135115	22.9	139822	14.4	116151	13.6
	14	105290	10.3	110385	15.7	111039	15.1
	15	60114	11.3	65517	15.8	67612	8.0
	Mean Positive	**188020**		**190141**		**191288**	
	Modulation	**22.2**		**23.1**		**21.1**	

2.4 Alkaline Phosphatase Stabilizer

Two commercial alkaline phosphatase stabilizers and the In-House AP stabilizer were tested as detection antibody (DAb) diluents. The In-House AP stabilizer was prepared by adding 0.1mM zinc chloride and 5mM magnesium chloride to the 3% BSA TBS blocking buffer. The IgG DAb concentration was tested at 50ng/ml in the respective buffers while capture concentration is at 1ug/ml. The protocol run does a final sample dilution of 1:25 along with a 10,10,10 minutes incubation time.

Pooled controls were prepared by pooling clinical samples that were determined to be either positive or negative based on the Diasorin Kit. The "Blank" refers to the control with only detection antibody and substrate added and no sample.

Table [SEQ Table * ARABIC]: Detection Antibody Stabilizers

DAb diluent	SAMPLE TYPE	Mean RLU	CV%	Modulation
3% BSA Assay Buffer	Pooled Pos	135363	16.6	11.8
	Pooled Neg	11435	10.0	
	BLANK	4823	9.4	
InHouse AP Stabilizer-3%BSA	Pooled Positive	181786	16.2	16.6
	Pooled Negative	10931	10.1	
	BLANK	902	4.6	
Stabilzyme	Pooled Positive	48821	22.3	14.0
	Pooled Negative	3496	22.7	
	BLANK	596	28.9	
Biostab	Pooled Positive	208327	21.6	14.2
	Pooled Negative	14686	21.4	
	BLANK	746	18.3	

2.5 Detection Antibody Titration

The AP conjugated detection antibody was titrated in In-House AP Stabilizer with a sample dilution of 1:25 and antigen coated at 1 ug/mL. The best modulation between the positive and negative pooled controls was observed at 5 ng/ml DAb. The "Blank" refers to the buffer control with only detection antibody and substrate added and no sample.

Table [SEQ Table * ARABIC]: Detection antibody titration

[Dab]ng/ml	SAMPLE TYPE	Mean RLU	CV%	Modulation
50	Pooled Positive	181786	16.2	16.6
	Pooled Negative	10931	10.1	
	BLANK	902	4.6	
25	Pooled Positive	118177	14.6	20.3
	Pooled Negative	5811	15.6	
	BLANK	463	18.0	
10	Pooled Positive	58156	7.3	21.7
	Pooled Negative	2675	12.8	
	BLANK	283	9.2	
5	Pooled Positive	30823	10.0	21.6
	Pooled Negative	1429	9.9	
	BLANK	238	14.4	

2.6 Sample Dilution

The effect of sample dilution was tested with final sample dilutions of 1:25 and 1:50 into 3% BSA blocking buffer. Antigen was 1 ug/ml and DAb was 5 ng/ml in In-house AP Stabilizer. Clinical samples were tested on the Diasorin Kit and then used as the test set on the Theranos system. Modulation between positive controls and negative control samples was best at 1:25. The protocol run includes a sample, detection conjugate and substrate incubation time of 10,10,10 minutes respectively.

Table [SEQ Table * ARABIC]: Sample dilution

		1:25			1:50		
Sample Type	Sample #	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
Negative	1	1493	16.1	23.0	893	19.8	18.3
	3	1219	8.6		714	13.2	
	4	1226	3.9		769	3.3	
	5	1163	14.2		710	13.0	
	6	817	11.2		517	21.5	
	Mean Neg	1183			721		
Positive	7	2998	26.5		1587	28.4	
	8	48115	9.7		24436	9.2	
	9	19776	6.6		10211	11.6	
	10	26183	15.0		12241	1.9	
	11	5189	18.1		2864	18.2	
	12	101022	14.6		48863	25.4	
	13	17227	30.0		7434	24.8	
	14	14788	6.9		6386	12.7	
	15	9164	7.4		4576	13.5	
	Mean Pos	27162			13177		
	Modulation	23			18		

2.7 Incubation Times

The effect of shorter reagent incubation times was tested with sample, detection conjugate and substrate incubation times of 10, 10. 10, 10, 10, 1, 5, 5, 5, 5, 5, 1 and 2, 2, 1 minutes. Assay modulation was good at the 5, 5, 5 incubation time and was used for further optimization.

Table [SEQ Table * ARABIC]: Incubation Time

Reagent Incubation (Min)	Sample Type	Mean RLU	CV%	Modulation
10, 10, 10	Pooled Positive	30823	10.0	21.6
	Pooled Negative	1429	9.9	
	BLANK	238	14.4	
10, 10, 1	Pooled Positive	7594	15.1	15.1
	Pooled Negative	503	10.3	
	BLANK	183	23.8	
5, 5, 5	**Pooled Positive**	**7577**	**11.8**	**14.9**
	Pooled Negative	**509**	**8.8**	
	BLANK	**193**	**5.6**	
5, 5, 1	Pooled Positive	2569	16.0	10.0
	Pooled Negative	257	10.8	
	Blank	153	6.2	
2, 2, 1	Pooled Positive	994	8.2	5.7
	Pooled Negative	174	15.1	
	Blank	137	14.7	

2.8 Clinical Samples Antigen 5

Clinical samples were tested on the Diasorin Kit and then tested on the Theranos system. Highlighted in red (Table 11B) are negative pediatric samples that seem to be false positives as they shower higher signal RLU similar to that of the low positive samples observed in Table 11A (highlighted in green).

Here, antigen concentration is 1 ug/ml while detection antibody is 5 ng/ml in In-house AP Stabilizer. The protocol run includes a sample dilution of 1:25 and a sample, detection conjugate and substrate incubation time of 5,5,5 minutes respectively.

Table [SEQ Table * ARABIC]: Clinical Samples -Antigen 5

A. Clinical Samples on the Theranos system

Negative	Clinical Samples	Sample ID	Mean RLU	CV%
	Set3	1	600	5.1
	Set4	50	356	8.5
	Set4	3	511	9.3
	Set4	4	519	12.3
		Mean	**496**	
Positive	Set2	1	15726	8.6
		2	1311	7.7
		3	14708	8.8
		5	24502	8.9
		6	1129	16.5
		8	6384	13.5
		10	8069	11.9
		12	1377	17.5
		15	27613	16.5
		20	4798	8.7
		21	3759	13.9
		22	11113	9.6
		28	1890	5.6
	Set3	2	31920	16.8
		Mean Positive	**26773**	
		Mod	**53.9**	

B. Negative Pediatric Samples (Set 5) run on the Diasorin Kit and the Theranos System

	Diasorin Kit Result		Theranos System Result	
Sample ID	Mean OD	CV%	Mean RLU	CV%
1	0.116	5.2	420	1.7
2	0.101	0.3	337	2.9
3	0.127	0.9	359	6.7
5	0.127	5.3	492	16.1
6	0.151	11.2	712	4.6
7	0.302	7.3	1214	15.1
9	0.135	3.2	827	12.8
11	0.129	5.9	925	9.2
12	0.137	25.3	3393	7.3
13	0.18	1.5	1551	9.5
14	0.132	20.4	544	11.9
15	0.11	7.9	453	8.1
16	0.142	3.9	2773	10.7

2.9 Blocking Buffers to Resolve False Positives -Antigen 5

Different blocking buffers were tested as sample diluents with the clinical samples that had shown a negative result in the Diasorin kit but a positive result in the Theranos assay. Heterophilic Blocking Reagent (HBR) by Scantibodies and the Surmodics Protein Free assay diluent had no beneficial effect on the assay. While some of these diluents did help reduce the false positive signal, they also reduced the true positive signal. The assay conditions included the antigen concentration at 1 ug/ml and detection antibody at 5 ng/ml in In-house AP Stabilizer. The protocol run includes a sample dilution of 1:25 and a sample, detection conjugate and substrate incubation time of 5, 5, 5 minutes respectively.

Table [SEQ Table * ARABIC]: Blocking Buffers -Antigen 5

		3% BSA in TBS		Surmodics Protein Free		HBR-400ug/ml	
Sample #		**Mean RLU**	**CV%**	**Mean RLU**	**CV%**	**Mean RLU**	**CV%**
Negative Samples	Set 4-#3	505	13.0	422	4.1	512	7.5
	Set 4-#4	512	16.4	311	8.3	419	9.8
Mean Negative		508		366		466	
False Positive	Set 5- 7	1620	12.8	717	7.9	1336	2.2
	Set 5-11	1031	5.5	779	10.5	1081	11.8
	Set 5-12	4528	19.2	1258	9.5	3165	21.2
	Set 5-13	2018	4.1	794	14.5	1595	12.7
	Set 5-16	4352	12.3	1028	6.4	4416	15.1
Mean of False Pos		**2710**		**915**		**2319**	
Positive Samples	Set2-2	1384	10.5	656	5.9	1146	11.5
	Set2-3	13743	14.4	8588	11.1	13399	18.3
	Set2-8	3193	113.1	4462	7.8	6601	4.6
	Set2-10	7752	16.1	3587	6.2	7397	12.0
	Set2-12	1865	12.8	991	10.5	1595	8.0
Mean Pos		5587		3657		6027	
Mean Pos/Mean Neg		11.0		10.0		12.9	

2.10 Coating Methods to Address False Positives

Different coating protocols were tested with a subset of the problem samples. The direct coating method with Antigen 6 seems to significantly decrease the false positive signal compared to the biotinylated Antigen 5 on avidin. Both antigen concentrations were tested at 10 ug/ml and detection antibody concentration at 5 ng/ml in In house AP Stabilizer. A manual 1:25 sample dilution was performed and the protocol was run at the 10,10, 10 minute incubation time to maximize modulation between positive and negative samples.

Table [SEQ Table * ARABIC]: Antigen Coating Methods to Resolve False Positives

Sample Type	**Sample #**	**Antigen 5 –avidin**		**Antigen 6 – Direct Coat**	
		Mean RLU	**CV%**	**Mean RLU**	**CV%**
Negative	Set 3-4	1225	7.9	633	9.9
False Positives	Set 5-7	5354	13.0	659	6.5
	Set5-11	3203	12.6	728	3.4
	Set5-13	5032	8.8	1257	7.4
Low Positive	2	2786	19.1	1266	9.0
	6	2905	4.1	2041	4.1
	12	4193	8.3	1335	4.5
	3	50727	20.8	6615	21.7

2.11 Blocking Buffers with Direct-Coat Antigen to Resolve False Positives

Different diluents were screened (Table 14) with a subset of problem samples to eliminate the false positive sample issue using the direct-coated Antigen 6. Starting Block from Pierce helped distinguish further between the negative and false positive problem samples.

A larger set of samples was tested with starting block as the diluent to confirm the resolution of false positives. The antigen concentration was 5 ug/ml while the detection antibody concentration was 50 ng/ml in In house AP Stabilizer. A manual 1:25 sample dilution was performed and the protocol was run at the 10,10,10 minute incubation time. All the negative samples showed a lower RLU response than all of the positive samples.

Table [SEQ Table * ARABIC]: Blocking Buffers with Direct-Coat Antigen to Resolve False Positives

		Diasorin Kit Diluent		Surmodics Protein Free		Starting Block		Sea Block		400ug/ml HBR	
Sample Type	Sample ID	Mean RLU	CV%	Mean RLU	CV%	Mean RLU	CV%	Mean RLU	CV%	Mean RLU	CV%
Negative	Set 4-4	705	8.2	1574	31.1	406	15.0	7697	15.2	703	14.6
False Pos	Set5-11	661	18.3	588	12.7	984	13.3	2364	12.1	5187	5.6
Positive	Set 2-2	3589	6.8	1965	4.1	6674	11.2	5863	6.0	10658	4.4
	Set2-10	1514	12.1	1212	15.8	4024	17.8	4480	18.2	12038	14.5
	Set2-28	1886	24.5	764	16.7	3515	15.5	3077	6.3	3580	11.2

Table [SEQ Table * ARABIC]: Starting Block Diluent with Direct-Coat Antigen to Resolve False Positives

Sample Type	Sample Set	Sample #	Mean RLU	CV%
Negative	Set 4	4	406	15.0
	Biorec-EBV Set	3	1736	23.6
	Set 5 (Pediatric)	7	1987	9.2
		11	984	13.3
		12	960	17.4
		13	2400	7.7
Positive	Set 2	2	6674	11.2
		10	4024	17.8
		28	3515	15.5
		3	33745	5.2
		6	5840	9.2
		8	37598	19.5
		12	9143	10.9
	Biorec-EBV	1	54104	9.6

		2	25286	15.1
		4	20851	11.5

2.12 Capture Surface Titration -Antigen 6

The capture surface Antigen #6 was titrated at the following concentrations: 5 ug/ml, 2.5 ug/ml and 1 ug/ml. The antigen was prepared in carbonate bicarbonate buffer and was directly coated onto the surface. A manual sample dilution of 1:25 was performed while detection antibody was 50 ng/ml in regular 3% BSA blocking buffer. The protocol was run at the 10,10,10 minute incubation time.

Similar modulation was observed for both 5 ug/ml and 2.5 ug/ml. However, the lower concentration of capture seemed to decrease the true signal for some of the low positive samples and was therefore not optimal.

Table [SEQ Table * ARABIC]: Capture Surface Titration - Antigen 6

Sample Type	Sample#		5 ug/ml Mean RLU	5 ug/ml CV%	2.5 ug/ml Mean RLU	2.5 ug/ml CV%	1 ug/ml Mean RLU	1 ug/ml CV%
Negative	Set 4	1	970	4.4	627	9.7	623	11.8
	Set 4	3	591	21.4	465	10.4	496	7.7
	Set 5	11	4651	6.1	4621	27.0	4997	8.8
	Set 5	13	11409	5.1	10951	5.1	11208	19.8
	Mean Neg		**4405**		**4166**		**4331**	
Positive Clinicals	Set 2	2	10953	4.6	7431	16.4	7692	15.1
		3	61415	10.8	65201	32.1	58634	13.8
		6	24278	17.3	27071	11.6	26156	12.2
		8	59936	10.6	48119	23.0	38587	16.6
		10	13509	11.3	9291	12.5	7745	8.0
		12	19145	12.3	19172	45.4	15620	5.4
		28	4365	8.2	4354	32.2	3321	22.4
	Mean Pos		**27657**		**25805**		**22537**	
	Modulation		**6.3**		**6.2**		**5.2**	

2.13 Clinical Samples - Antigen 6

A large set of clinical samples were tested on the Diasorin Kit, and results were compared to the Theranos System. The cut-off criteria for the Diasorin kit is listed in the table while a general cutoff value for the Theranos system was determined by taking the mean RLU of the negative samples plus 2.5 times the standard deviation of the negative samples. The assay conditions included 5 ug/ml of Antigen 6 directly coated on the surface, 50 ng/ml of detection antibody in In-House AP Stabilizer and Starting Block as the sample diluent. The protocol performed a 1:25 final sample dilution along with a 10,10,10 minute incubation period.

Table [SEQ Table * ARABIC]: Clinical Samples -Antigen 6

Green = Negative Result
Pink = Positive Result

			Theranos		Diasorin Kit		
SAMPLE TYPE	**SAMPLE SET**	**ID #**	**Mean RLU**	**CV%**	**Mean OD**	**CV%**	**Cutoff Calibrator Diasorin Kit**
Negative Samples	Set3-	1	809	8.2	0.11	7.6	Positive ≥OD: 0.324
		4	2339	12.8	0.167	6.9	
	Set 1	#3	530	13.0	0.171	0.4	Positive ≥ OD: 0.354
	Set 1	#7	370	22.1	0.142	10.2	
	Biorec -EBV Neg	3	2110	10.1	0.137	2	Positive ≥OD: 0.316
	Pediatric Set-Set 5	1	813	4.0	0.116	5.2	Positive ≥OD: 0.316
		2	615	5.9	0.101	0.3	
		3	602	13.1	0.127	0.9	
		5	1375	18.8	0.127	5.3	
		6	2223	19.2	0.151	11.2	
		7	1628	24.7	0.302	7.3	
		9	3248	23.1	0.135	3.2	
		11	1480	3.0	0.129	5.9	
		12	788	14.7	0.137	25.3	
		13	3373	11.0	0.18	1.5	
		14	2695	13.8	0.132	20.4	
		15	584	9.9	0.11	7.9	
		16	1176	9.6	0.142	3.9	
	Mean Negative RLU		1505				
Nominal Cutoff (2.5*StDev)+Mean RLU							
Positive Samples	Biorec-EBV Set	1	99202	21.2	1.056	1.2	Positive ≥OD: 0.316
		2	35350	15.3	0.646	6	
		4	27474	12.3	0.545	2.7	
		5	50881	19.6	0.722	6.1	
		8	236330	13.8	3.674	1.6	
		9	79982	9.2	3.457	0.3	
		10	64277	19.4	3.53	1	
	Set 2	1	35553	16.9	3.198	2.3	Positive ≥OD: 0.108
		2	11871	9.0	1.056	2.3	
		3	54475	14.1	2.716	2	
		5	377897	17.9	3.402	2.2	
		6	11334	9.4	1.457	2.2	
		10	6272	14.0	0.486	6.3	
		13	22635	4.8	1.287	0.5	
		15	8270	17.0	2.986	2.1	
		18	59079	4.4	2.703	1.1	
		21	15788	17.2	1.371	1.3	
		28	4203	22.6	3.135	0.3	
	Set3	2	120134	9.2	3.331	0.4	Positive ≥ OD: 0.324
		3	183419	23.6	3.317	0.6	
		15	14676	6.1	0.578	0.6	
		20	11747	20.3	0.704	14.8	
		21	2841	18.7	0.354	11.6	
	Mean Positive RLU		66682				
	Modulation		44.3				

2.14 Specificity - Antigen 6

Positive disease samples known to cause false positives in this EBV IgG assay were tested on the Theranos system. Both rheumatoid factor (RF) and Human Anti-Mouse Antibodies (HAMA) positive samples were tested on the Theranos system. Since most adults are positive for EBV IgG, it was expected that these samples would be positive in the assays. Data obtained on the Diasorin Kit was comparable to the data obtained from the Theranos system with the exception of 1 out of 10 RF samples. Similarly, quality controls for CMV, HSV and VZV correlated between the Diasorin and the Theranos system. Antigen6 was coated at 5 ug/ml directly on the surface and DAb was at 50ng/ml in In House AP stabilizer. The protocol run performed a 1:25 final sample dilution along with a 10, 10, 10 minute incubation period.

Table [SEQ Table * ARABIC]: Specificity -Antigen 6

		Theranos		Diasorin Kit		
Sample Type	ID#	Mean RLU	CV%	Mean OD	CV%	Criteria- Diasorin Kit
RF Samples	1	1833	14.4	2.791	3.9	Positive ≥OD: 0.321
	2	7503	21.1	0.21	0.1	
	3	57894	14.0	3.244	1.4	
	4	343339	18.3	3.657	1.1	
	5	318486	12.1	3.635	0.6	
	6	154042	13.1	3.56	1.4	
	7	170814	45.9	3.589	1.9	
	8	73852	10.4	3.468	1.4	
	9	77431	12.5	2.479	3.5	
	10	260030	9.7	3.554	2.7	
HAMA Samples	1	98502	16.5	3.507	1.7	
	2	12361	12.9	0.648	0	
	3	319785	9.1	3.422	1.8	
	4	106267	12.6	3.013	1.7	
	5	20052	23.1	3.306	1.6	
	6	59021	14.9	3.54	3.5	
	7	121852	16.2	3.539	3.7	
	8	218637	4.6	3.84	5.9	
	9	126522	16.0	3.541	1.4	
	10	59219	13.8	3.551	0.5	
CMV	QC	109428	16.4	3.209	0.3	
HSV	QC	54431	14.9	1.944	3.7	
VZV	Kit	22603	11.0	0.507	0.2	
Mean Negative Clinicals *		**1505**				
Mean Positive Clinicals*		**66682**				

*Refer to Previous Table

2.15 Stability Studies

Stability monitoring is ongoing for the the assay reagents stored at 4°C and protected from light. Different detection antibody stabilizers will be evaluated throughout this study. These include detection antibody in either Stabilzyme AP or the Theranos In-house AP stabilizer.