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Herpes Simplex Virus 2 IgG Assay Feasibility Report

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

An enzyme linked immunosorbent assay (ELISA) was developed for the qualitative detection of Herpes Simplex Virus type 2 (HSV2) IgG Antibody. Herpes Simplex Virus Type 1 (HSV1) primarily infects the mouth and nose while HSV2 primarily infects the genital area. Both are characterized by blistering of the epithelia and a dormant phase in the ganglion of sensory neurons. Primary infections of HSV may lead to illness during pregnancy and compromise fetal and newborn health. HSV1 and HSV2 have similar epitope structures, and it is important to differentiate between them. This report describes the assay development and performance of the HSV2 IgG assay as aid to determining immune status. The Theranos assay is designed to detect anti HSV2 IgG in human whole blood, serum and plasma.

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

The Liaison HSV-2 IgG test available in the CLIA lab was the predicate method. A secondary method was used for confirmation of results.

- Liaison HSV-2 IgG , Cat.310620
- EuroImmun HSV-2 (gG2) ELISA (IgG), Cat. EI 2531-9601-24 G

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

The HSV-2 IgG ELISA was developed using the HSV-2 gG1M recombinant antigen as the capture surface. Human IgG antibodies in serum or plasma samples bind specifically to the HSV-2 antigen for 5 minutes followed by a wash cycle. Bound human IgG anti-HSV-2 antibodies are detected using an AP labeled mouse monoclonal antibody against human IgG. After incubation with the detector antibody for 5 minutes, another wash cycle was performed and the alkaline phosphatase substrate is incubated on the surface for another 5 minutes. The resulting chemiluminescence is read in relative light units (RLU) on the Theranos system.

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Herpes Simplex Virus type 2 gG antigen	Virusys	H2V134-1
Mouse Anti-hIgG clone 2C11	Novus	NB100-2046
Alkaline Phosphatase Labeling Kit (SH)	Dojindo	LK13-10
Theranos Substrate	Theranos	In House
Assay Diluent (Protein Free)	Surmodics	SM01-1000
Theranos AP Conjugate Stabilizer	Theranos	In House
Tris Buffered Saline with Tween 20	Sigma	T9039-10PAK
Theranos Cartridge	Theranos	In House
Theranos System	Theranos	In House

2 ASSAY DEVELOPMENT

2.1 Antigen Screen

Antigens C21-C28 were tested on micro titer plates to determine the best antigen captures to move forward with onto the Theranos system. The antigens were coated directly onto the plate using a 30 minute incubation of antigen at 5ug/mL in Carbonate-Bicarbonate buffer and 10 minute incubation of the house fixative buffer. A 10-10-10 incubation protocol was used with 100ng/mL AP-conjugated Novus clone 2C11 antibody used for the detection. Three negative and three positive samples were tested and the average RLU of the negatives was used to determine the modulation of each sample. A modulation cutoff of 1.5 was used to narrow down the best candidates for further testing. Captures C21, C26, and C27 performed best.

Table [SEQ Table * ARABIC]: MTP Initial Antigen Capture

		C21			C22		
Sample Type	Liaison Result	Avg	%CV	Modulation	Avg	%CV	Modulation
HSV2	Positive	1769244	10	36.24	149375	17	6.81
HSV2	Positive	297278	8	6.09	105423	4	4.81
Negative	Negative	72830	38	1.49	38473	34	1.75
BioRad Pos	Positive	1028591	29	21.07	98294	6	4.48
BioRad Neg	Negative	52520	11	1.08	15184	17	0.69
Blocking Buffer	Negative	21129	39	0.43	12160	14	0.55
Average of negatives		48826			21939		
		C23			C24		
Sample Type	Liaison Result	Avg	%CV	Modulation	Avg	%CV	Modulation
HSV2	Positive	1460765	20	30.04	1655510	13	35.01
HSV2	Positive	681677	32	14.02	985053	1	20.83
Negative	Negative	80379	28	1.65	88103	20	1.86
BioRad Pos	Positive	612956	1	12.61	653315	24	13.82
BioRad Neg	Negative	28255	21	0.58	28524	40	0.60
Blocking Buffer	Negative	37226	52	0.77	25243	25	0.53
Average of negatives		48620			47290		
		C25			C26		
Sample Type	Liaison Result	Avg	%CV	Modulation	Avg	%CV	Modulation
HSV2	Positive	483967	7	13.05	1620842	20	29.63
HSV2	Positive	242732	5	6.55	923343	2	16.88
Negative	Negative	21072	36	0.57	44618	30	0.82
BioRad Pos	Positive	223527	11	6.03	533991	0	9.76
BioRad Neg	Negative	43462	11	1.17	64863	52	1.19
Blocking Buffer	Negative	46717	27	1.26	54630	14	1.00
Average of negatives		37084			54704		
		C27			C28		
Sample Type	Liaison Result	Avg	%CV	Modulation	Avg	%CV	Modulation
HSV2	Positive	830721	17	17.27	1535704	4	34.37
HSV2	Positive	800169	23	16.63	1139688	5	25.51
Negative	Negative	39863	12	0.83	89425	37	2.00
BioRad Pos	Positive	486874	18	10.12	588442	22	13.17
BioRad Neg	Negative	36524	40	0.76	23960	20	0.54
Blocking Buffer	Negative	67955	1	1.41	20643	10	0.46
Average of negatives		48114			44676		

2.2 Theranos Antigen Capture Finalization

Antigens C21, C26, and C27 were tested to determine which would work best as the antigen capture. All are different segments of the recombinant HSV-2 gG antigen. The Theranos tips were coated directly with each capture in Carbonate-Bicarbonate buffer at 5ug/mL. The assay was performed using the reader protocol Generic2_10X which is a 10-10-10 incubation protocol with a 10x sample dilution. The AP-conjugated Novus clone 2C11 antibody was used at 100 ng/mL in the House Blocking Buffer as the detection antibody. C26 performed best and is used in further testing. Samples were run in triplicate with two tips per cartridge.

The average and standard deviation were calculated using the response of the negative samples. The cutoff was determined by the following equation:

$$Stdev*2+Avg=Theranos\ Cutoff$$

where stdev and avg(-) are calculated using the negative samples within that condition

To determine the Antibody Index the following equation was used:

$$Avg\ RLU/Theranos\ Cutoff= Antibody\ Index$$

(samples that were HSV2 negative but HSV1 positive were excluded from the average and standard deviation calculations of the negative samples) Green (below 0.9 Index) is considered a negative sample. Red (above 1.1 Index) is considered a positive sample. Yellow (between 0.9 and 1.1) is considered an equivocal sample.

Table [SEQ Table * ARABIC]: Antigen Capture

Liaison Result	Sample ID	C21			C26			C27		
		Mean	CV %	Theranos Index	Mean	CV %	Theranos Index	Mean	CV %	Theranos Index
Negative	N1	26355	11	0.03	425	21	0.04	701	22	0.08
Negative	N2	896265	6	0.86	401	25	0.04	1000	31	0.12
Negative	N4	45700	22	0.04	684	9	0.07	4527	35	0.54
Negative	N5	76360	19	0.07	11185	14	1.16	7463	67	0.89
Negative	N6	97346	12	0.09	2285	13	0.24	1071	25	0.13
Negative	N7	23114	7	0.02	1028	6	0.11	1753	15	0.21
Negative	N8	13864	18	0.01	580	18	0.06	3819	13	0.46
Negative	N10	895088	9	0.86	2305	18	0.24	5869	6	0.70
HSV1pos	11	244095	7	0.23	7991	18	0.83	17539	21	2.10
HSV1pos	16	790497	6	0.76	18178	16	1.88	23402	29	2.80
HSV1pos	35	58978	19	0.06	22332	6	2.31	26932	27	3.22
HSV1pos	44	203077	17	0.19	5406	12	0.56	5816	4	0.70
HSV1pos	45	212350	9	0.20	10467	11	1.08	11415	13	1.37
Positive	N9	727724	17	0.70	254085	15	26.30	180219	39	21.58
Positive	2	343607	9	0.33	83901	14	8.69	61786	50	7.40
Positive	4	402984	20	0.38	91091	18	9.43	65765	7	7.87
Positive	7	689277	11	0.66	68355	9	7.08	56952	27	6.82
Positive	9	134962	12	0.13	9979	9	1.03	13464	31	1.61
Positive	15	233391	6	0.22	22001	13	2.28	15660	13	1.87
Positive	17	266171	5	0.25	41916	26	4.34	37506	37	4.49
Positive	18	372000	10	0.36	88766	19	9.19	148738	11	17.81
Positive	41	86282	17	0.08	23293	7	2.41	34212	13	4.10
Positive	43	115175	18	0.11	43886	18	4.54	28516	7	3.41
		Avg(-)	stdev(-)	Avg(-)+ Stdev(-)	Avg(-)	stdev(-)	Avg(-)+ Stdev(-)	Avg(-)	stdev(-)	Avg(-)+ Stdev(-)
		259261	393802	1046865	2362	3649	9660	3275	2539	8352

2.3 Capture Surface Titration

Optimum surface coating condition was determined by titrating the capture surface. Tips were coated with C26 antigen at 10 ug/mL, 5 ug/mL and 2.5 ug/mL. The assay was performed using the Generic2_10x protocol on the 3.0 reader. The detector antibody was used at 100 ng/mL. A coating concentration of 5ug/mL gave the lowest %CVs and background, and also a good difference between positive and negative samples. Future experiments used 5ug/mL capture coating. Samples were run in triplicate with two tips per cartridge.

The following equations was used for sample analysis

Assay Cut-off = Average RLU of confirmed negative samples+ 5* Std deviation
Avg RLU/Assay Cut-off= Antibody Index

Where stdev(-) and avg(-) are calculated using the negative samples within that condition

(samples that were HSV2 negative but HSV1 positive were excluded from the average and standard deviation calculations of the negative samples) Green (below 0.9 Index) is considered a negative sample. Red (above 1.1 Index) is considered a positive sample. Yellow (between 0.9 and 1.1) is considered an equivocal sample.

Table [SEQ Table * ARABIC]: Capture Titration of C13

Sample ID	HSV2 IgG Liason Result	10ug/mL Mean RLU	CV %	Theranos Index	5ug/mL Mean RLU	CV %	Theranos Index	Theranos Result	2.5ug/mL Mean RLU	CV %	Theranos Index
BRN	Negative	635	32	0.09	608	22	0.12	Negative	496	26	0.07
SCP	Negative	1699	37	0.24	1266	23	0.26	Negative	1539	23	0.22
LN4	Negative	872	27	0.12	620	23	0.13	Negative	463	30	0.07
LN5	Negative	968	25	0.14	592	26	0.12	Negative	654	22	0.09
EN9	Negative	624	24	0.09	632	29	0.13	Negative	1048	42	0.15
EN10	Negative	684	25	0.10	485	28	0.10	Negative	669	30	0.10
Z19 (HSV1)	Negative	976	23	0.14	1241	30	0.25	Negative	1304	23	0.19
Z34 (HSV1)	Negative	3222	16	0.46	3066	11	0.62	Negative	2681	10	0.38
BRP	Positive	12065	24	1.71	13273	20	2.69	Positive	14266	2	2.04
Z22	Positive	38553	17	5.47	49430	24	10.03	Positive	54619	14	7.80
Z37	Positive	62530	12	8.87	90823	25	18.42	Positive	76870	31	10.98
Z38	Positive	142215	13	20.18	268421	27	54.45	Positive	163985	16	23.41
Z40	Positive	87934	12	12.48	117826	29	23.90	Positive	107912	29	15.41
RF06	Positive	141121	10	20.02	226010	28	45.84	Positive	160884	12	22.97
HA05	Positive	73376	17	10.41	90000	26	18.26	Positive	78995	23	11.28
Experimental Cutoff		Avg RLU	stdev(-)	Cutoff	Avg(-)	stdev(-)	Cutoff		Avg(-)	stdev(-)	Cutoff
		914	409	7048	700	282	4930		812	413	7004

2.4 Assay Diluent

The effect of the Assay Diluent was tested using House Blocking Buffer and Protein free Assay Diluent. The assay was performed using the Generic2_10x protocol and 5ug/mL tip coating of C26. The detection was diluted to 100ng/mL in the Theranos House stabilizer. The Protein free assay Diluent performed best with the lowest background, best separation of negatives and positives, and the best clinical correlation to the predicate method. Samples were run in triplicate with two tips per cartridge.

The following equations was used for sample analysis

Assay Cut-off = Average RLU of confirmed negative samples+ 5* Std deviation

Avg RLU/Assay Cut-off= Antibody Index

where stdev(-) and avg(-) are calculated using the negative samples within that condition

(samples that were HSV2 negative but HSV1 positive were excluded from the average and standard deviation calculations of the negative samples) Green (below 0.9 Index) is considered a negative sample. Red (above 1.1 Index) is considered a positive sample. Yellow (between 0.9 and 1.1) is considered an equivocal sample.

Table [SEQ Table * ARABIC]: House Blocking Buffer and Surmodics Protein Free Assay Diluent

Sample ID	Liason Result	EuroImmun Result	Blocking Buffer			Protein Free			Theranos Result
			Mean	CV %	Theranos Index	Mean	CV %	Theranos Index	
BRN	Negative	Negative	675	26	0.02	758	5	0.28	Negative
SCP	Negative	Negative	19117	21	0.49	894	10	0.33	Negative
EN4	Negative	Negative	1779	21	0.05	742	29	0.27	Negative
EN5	Negative	Negative	1470	23	0.04	961	27	0.35	Negative
EN9	Negative	Negative	587	20	0.01	780	15	0.28	Negative
EN10	Negative	Negative	1844	26	0.05	1073	27	0.39	Negative
Z19 (HSV1)	Negative	Negative	10475	19	0.27	1192	35	0.43	Negative
Z34 (HSV1)	Negative	Negative	9445	12	0.24	1778	16	0.65	Negative
BRP	Positive	Negative	44048	36	1.12	17151	21	6.25	Positive
Z22	Positive	Equivocal	86162	7	2.19	40687	20	14.83	Positive
Z37	Positive	Positive	116263	27	2.96	51291	22	18.69	Positive
Z38	Positive	Positive	231321	18	5.89	131386	17	47.88	Positive
Z40	Positive	Positive	142181	13	3.62	70978	24	25.86	Positive
RF06	Positive	Positive	110843	19	2.82	61965	15	22.58	Positive
HA05	Positive	Positive	189162	18	4.81	142499	33	51.92	Positive
Experimental Cutoff			Avg RLU	stdev(-)	Cutoff	Avg(-)	stdev(-)	Cutoff	
			5674	6723	39289	1022	344	2744	

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2.5 Fixative Buffer

The effect of varying the Fixative Buffer on the assay was evaluated by testing the following: House Blocking Buffer, Protein free, Sea Block, StartingBlock, and SuperBlock. The assay was performed using the Generic2_10x protocol and 5ug/mL tip coating of C26. The detection was diluted to 100ng/mL in the Theranos House stabilizer. The Protein free assay Diluent will be used in future tips due to the higher modulation between negative and positive samples. Samples were run in triplicate with two tips per cartridge.

To determine the Theranos Index the following equations were used:
Assay Cut-off = Average RLU of confirmed negative samples+ 5* Std deviation
Avg RLU/Assay Cut-off= Antibody Index

where stdev(-) and avg(-) are calculated using the negative samples within that condition

(samples that were HSV2 negative but HSV1 positive were excluded from the average and standard deviation calculations of the negative samples) Green (below 0.9 Index) is considered a negative sample. Red (above 1.1 Index) is considered a positive sample. Yellow (between 0.9 and 1.1) is considered an equivocal sample.

Table [SEQ Table * ARABIC]: Fixative Buffers: Blocking Buffer, Protein Free

Sample ID	Liason Result	EuroImmun Index	Blocking Buffer			Protein Free			
			Mean RLU	CV %	Theranos Index	Mean RLU	CV %	Theranos Index	Theranos Result
BRN	Negative	Negative	490	21	0.08	892	23	0.20	Negative
SCP	Negative	Negative	1418	27	0.23	1163	27	0.26	Negative
EN4	Negative	Negative	756	27	0.12	997	43	0.22	Negative
EN5	Negative	Negative	1126	24	0.18	897	23	0.20	Negative
EN9	Negative	Negative	649	10	0.11	675	20	0.15	Negative
EN10	Negative	Negative	635	7	0.10	1338	20	0.30	Negative
Z19 (HSV1)	Negative	Negative	1197	28	0.19	852	20	0.19	Negative
Z34 (HSV1)	Negative	Negative	3039	13	0.49	3113	10	0.70	Negative
BRP	Positive	Negative	15721	18	2.56	24158	18	5.40	Positive
Z22	Positive	Equivocal	72532	10	11.81	90481	9	20.24	Positive
Z37	Positive	Positive	97253	15	15.83	110463	18	24.71	Positive
Z38	Positive	Positive	205946	15	33.53	228360	20	51.07	Positive
Z40	Positive	Positive	121006	19	19.70	133303	16	29.81	Positive
RF06	Positive	Positive	229525	16	37.37	168955	14	37.79	Positive
HA05	Positive	Positive	133296	22	21.70	117486	14	26.28	Positive
Experimental Cutoff			Avg	stdev	Cutoff	Avg	stdev	*Cutoff	
			846	353	6142	994	232	4471	

Table [SEQ Table * ARABIC]: Fixative Buffers: SeaBlock, StartingBlock, SuperBlock

Sample ID	Liason Result	EuroImmun Index	SeaBlock			StartingBlock			SuperBlock		
			Mean	CV %	Theranos Index	Mean	CV %	Theranos Index	Mean	CV %	Theranos Index
BRN	Negative	Negative	803	35	0.11	315	32	0.06	648	52	0.08
SCP	Negative	Negative	2013	15	0.27	1089	28	0.22	1777	47	0.23
EN4	Negative	Negative	1270	20	0.17	342	8	0.07	717	40	0.09
EN5	Negative	Negative	1049	22	0.14	601	14	0.12	838	31	0.11
EN9	Negative	Negative	1203	17	0.16	713	26	0.15	553	34	0.07
EN10	Negative	Negative	1342	20	0.18	522	27	0.11	1005	39	0.13
Z19 (HSV1)	Negative	Negative	1644	26	0.22	1184	27	0.24	1968	17	0.26
Z34 (HSV1)	Negative	Negative	3200	21	0.43	1720	21	0.35	2417	12	0.32
BRP	Positive	Negative	15296	9	2.07	13057	24	2.68	15311	9	2.01
Z22	Positive	Equivocal	70123	9	9.49	43377	13	8.90	52372	15	6.87
Z37	Positive	Positive	69838	26	9.45	60114	13	12.34	82772	18	10.85
Z38	Positive	Positive	182325	20	24.68	135364	17	27.79	155718	15	20.42
Z40	Positive	Positive	92875	13	12.57	97904	27	20.10	96813	17	12.69
RF06	Positive	Positive	163202	26	22.09	101757	17	20.89	101991	17	13.37
HA05	Positive	Positive	94564	10	12.80	79220	22	16.26	95028	12	12.46
Experimental Cutoff			Avg(-)	stdev(-)	*15Cutoff	Avg(-)	stdev(-)	*15Cutoff	Avg(-)	stdev(-)	*15Cutoff
			1280	407	7387	597	285	4871	923	447	7627

2.6 Effect of Sample Dilutions

Sample dilutions at 10x and 25x were tested to determine the effect of sample dilution on assay response. Both dilutions gave good correlation with the predicate method and acceptable modulation. Further tests were run at a 25X dilution. Samples were run in triplicate with two tips per cartridge.

To determine the Theranos Index the following equation was used:

$$\text{Avg RLU}/(\text{stdev}(-)*15+\text{avg}(-)) = \text{Theranos Index}$$

where stdev(-) and avg(-) are calculated using the negative samples within that condition

(samples that were HSV2 negative but HSV1 positive were excluded from the average and standard deviation calculations of the negative samples) Green (below 0.9 Index) is considered a negative sample. Red (above 1.1 Index) is considered a positive sample. Yellow (between 0.9 and 1.1) is considered an equivocal sample.

Table [SEQ Table * ARABIC]: Sample Dilutions

Sample ID	Liason Result	EuroImmun Result	1:10 Sample Dilution			1:25 Sample Dilution			Theranos Result
			Mean RLU	CV %	Theranos Index	Mean RLU	CV %	Theranos Index	
BRN	Negative	Negative	747	28	0.07	936	34	0.12	Negative
SCP	Negative	Negative	2139	17	0.21	1929	27	0.26	Negative
EN4	Negative	Negative	586	30	0.06	1253	12	0.17	Negative
EN5	Negative	Negative	718	7	0.07	1005	39	0.13	Negative
EN9	Negative	Negative	1193	24	0.12	902	22	0.12	Negative
EN10	Negative	Negative	576	23	0.06	733	26	0.10	Negative
Z19 (HSV1)	Negative	Negative	2697	27	0.27	1709	38	0.23	Negative
Z34 (HSV1)	Negative	Negative	7167	16	0.71	5617	23	0.74	Negative
BRP	Positive	Negative	49016	22	4.87	32259	9	4.27	Positive
Z22	Positive	Equivocal	161040	16	16.00	125191	23	16.59	Positive
Z37	Positive	Positive	238489	15	23.70	171540	25	22.73	Positive
Z38	Positive	Positive	281319	15	27.95	332555	8	44.06	Positive
Z40	Positive	Positive	299331	14	29.74	244400	27	32.38	Positive
RF06	Positive	Positive	351625	19	34.94	580002	12	76.85	Positive
HA05	Positive	Positive	236828	25	23.53	305510	18	40.48	Positive
Experimental Cutoff			Avg RLU	stdev(-)	Cutoff	Avg RLU	stdev(-)	Cutoff	
			993	605	10064	1126	428	7548	

2.7 Assay Protocols

The optimum assay protocol was determined by testing three protocols (10-10-10, 10-10-10 with PSW, and 5-5-5 with PSW) on the Theranos system. The assay conditions finalized from earlier experiments were used to test this. The 25x_PSW_5-5-5 gave the good modulation and low background. Because it also has a shorter assay run time, and also matches the HSV1 assay protocol, the 25X_PSW_5-5-5 was used moving forward. Samples were run in triplicate with two tips per cartridge.

To determine the Theranos Index the following equation was used:

$$\text{Avg RLU}/(\text{stdev}(-)*25+\text{avg}(-))=\textbf{Antibody Index}$$

where stdev(-) and avg(-) are calculated using the negative samples within that condition

(samples that were HSV2 negative but HSV1 positive were excluded from the average and standard deviation calculations of the negative samples) Green (below 0.9 Index) is considered a negative sample. Red (above 1.1 Index) is considered a positive sample. Yellow (between 0.9 and 1.1) is considered an equivocal sample.

Table [SEQ Table * ARABIC]: Assay Incubation and Post Sample Washing

Sample ID	Liason Index	EuroImmun Index	10-10-10 Mean RLU	CV %	Theranos Index	PSW10-10-10 Mean RLU	CV %	Theranos Index	PSW5-5-5 Mean RLU	CV %	Theranos Index	Theranos Result
BRN	Negative	Negative	791	22	0.08	626	32	0.03	745	17	0.12	Negative
SCP	Negative	Negative	1632	24	0.16	2299	3	0.12	1023	20	0.16	Negative
EN4	Negative	Negative	724	37	0.07	802	29	0.04	381	22	0.06	Negative
EN5	Negative	Negative	1353	19	0.13	1478	24	0.08	835	33	0.13	Negative
EN9	Negative	Negative	1333	11	0.13	2353	23	0.12	614	10	0.10	Negative
EN10	Negative	Negative	952	46	0.09	1360	24	0.07	527	19	0.08	Negative
Z19	Negative	Negative	1854	21	0.18	5514	70	0.28	580	22	0.09	Negative
Z34	Negative	Negative	18544	39	1.82	31595	20	1.61	6403	23	1.00	Equivocal
BRP	Positive	Negative	63701	12	6.26	243477	19	12.40	50171	13	7.82	Positive
Z22	Positive	Equivocal	242388	28	23.83	562006	17	28.62	118350	19	18.45	Positive
Z37	Positive	Positive	301457	25	29.63	798036	13	40.64	199990	13	31.17	Positive
Z38	Positive	Positive	575943	23	56.62	1242448	5	63.27	410207	16	63.94	Positive
Z40	Positive	Positive	396752	19	39.00	976922	12	49.75	285594	9	44.51	Positive
RF06	Positive	Positive	467365	15	45.94	1352784	13	68.89	500995	17	78.09	Positive
HA05	Positive	Positive	317869	16	31.25	929297	12	47.33	204768	10	31.92	Positive
Experimental Cutoff			Avg RLU	stdev(-)	*25 Cutoff	Avg RLU	stdev(-)	Cutoff	Avg RLU	stdev(-)	Cutoff	
			1131	362	10173	1486	726	19636	687	229	6416	

2.8 Detector Antibody Titration

To determine the optimum concentration of Novus 2C11 detector antibody, the assay was run using the best current conditions (25x_PSW_5-5-5). The detector antibody was titrated to 100ng/mL, 50ng/mL, and 25ng/mL. The best detector antibody concentration was determined to be 25ng/mL because of the lower CVs and large difference between negative and positive samples. Samples were run in triplicate with two tips per cartridge.

To determine the Theranos Index the following equation was used:

$$\text{Avg RLU}/(\text{stdev}(-)*25+\text{avg}(-))$$

where stdev(-) and avg(-) are calculated using the negative samples within that condition

(samples that were HSV2 negative but HSV1 positive were excluded from the average and standard deviation calculations of the negative samples) Green (below 0.9 Index) is considered a negative sample. Red (above 1.1 Index) is considered a positive sample. Yellow (between 0.9 and 1.1) is considered an equivocal sample.

Table [SEQ Table * ARABIC]: Detection Antibody Titration

Sample ID	Liason Index	EuroImmun Index	100ng/mL			50ng/mL			25ng/mL			Theranos Result
			Mean	CV %	Theranos Index	Mean	CV %	Theranos Index	Mean	CV %	Theranos Index	
BRN	Negative	Negative	473	52	0.14	241	52	0.08	120	10	0.08	Negative
SCP	Negative	Negative	710	18	0.21	467	36	0.16	240	16	0.16	Negative
EN4	Negative	Negative	371	43	0.11	168	24	0.06	152	23	0.10	Negative
EN5	Negative	Negative	523	25	0.16	271	29	0.09	205	24	0.14	Negative
EN9	Negative	Negative	485	31	0.15	315	27	0.11	248	25	0.16	Negative
EN10	Negative	Negative	469	28	0.14	187	31	0.06	150	19	0.10	Negative
Z19	Negative	Negative	410	34	0.12	290	36	0.10	133	36	0.09	Negative
BRP	Positive	Negative	21192	9	6.39	10050	21	3.36	8256	17	5.50	Positive
Z22	Positive	Equivocal	49986	21	15.08	24377	31	8.15	23536	31	15.67	Positive
Z37	Positive	Positive	92172	16	27.80	53977	17	18.05	28695	29	19.10	Positive
Z38	Positive	Positive	160502	36	48.41	119600	21	40.00	83053	21	55.29	Positive
Z40	Positive	Positive	151721	17	45.76	95301	21	31.87	60893	24	40.54	Positive
RF06	Positive	Positive	332218	25	100.21	229248	24	76.67	192928	22	128.43	Positive
HA05	Positive	Positive	153076	11	46.17	63542	15	21.25	53399	12	35.55	Positive
			Avg(-)	stdev(-)	*25Cutoff	Avg(-)	stdev(-)	*25Cutoff	Avg(-)	stdev(-)	*25Cutoff	
			505	112	3315	275	109	2990	186	53	1502	

2.9 Cutoff Determination

To determine the Cutoff (CO) value, 25 samples that were confirmed negative on the predicate method were used. The average RLU value and standard deviation of these 25 negative samples was used to calculate the cutoff and the Theranos Index. The cutoff was calculated to be an RLU 1069 and was used to determine test results in all following experiments. Samples were run in triplicate with two tips per cartridge.

The calculation of the CO was determined using the following:

Cutoff =1069 = Mean RLU + (Standard Deviation)*35

The Theranos index was calculated by using the established cutoff and the following equation:

RLU/Cutoff=Theranos Index

Green (below 0.9 Index) is a considered a negative sample. Red (above 1.1 Index) is considered a positive sample. Yellow (between 0.9 and 1.1) is considered an equivocal sample.

Table [SEQ Table * ARABIC]: Assay Cutoff Determination

Sample ID	Liaison Result	EuroImmun Result	Theranos Index	Theranos Result
LN01	Negative	Not Tested	0.15	Negative
LN02	Negative	Not Tested	0.15	Negative
LN03	Negative	Not Tested	0.17	Negative
EN04	Negative	Negative	0.14	Negative
EN05	Negative	Negative	0.15	Negative
EN09	Negative	Negative	0.13	Negative
EN10	Negative	Negative	0.18	Negative
EN11	Negative	Negative	0.16	Negative
EN15	Negative	Negative	0.23	Negative
EN16	Negative	Negative	0.11	Negative
EN17	Negative	Negative	0.18	Negative
EN21	Negative	Negative	0.18	Negative
EN23	Negative	Negative	0.14	Negative
EN25	Negative	Negative	0.15	Negative
EN26	Negative	Negative	0.15	Negative
EN27	Negative	Negative	0.16	Negative
EN28	Negative	Negative	0.19	Negative
M01	Negative	Not Tested	0.15	Negative
M02	Negative	Not Tested	0.12	Negative
M03	Negative	Not Tested	0.16	Negative
M04	Negative	Not Tested	0.17	Negative
F01	Negative	Not Tested	0.15	Negative
F02	Negative	Not Tested	0.15	Negative
F04	Negative	Not Tested	0.17	Negative
F05	Negative	Not Tested	0.17	Negative

2.10 Hematocrit and anti-coagulant Effects

The hematocrit effect was tested by comparing results from whole blood and its matching plasma in EDTA anti-coagulant. Five male and 5 female samples were used. The hematocrit effect was within a normal range. To test the effect of anti-coagulant, matched Li heparin, EDTA and serum samples were tested. The effects of anti-coagulants were negligible. Samples were run in triplicate with two tips per cartridge.

The Theranos index was calculated by using the established cutoff and the following equation:

RLU/1069=Theranos Index

Green (below 0.9 Index) is a considered a negative sample. Red (above 1.1 Index) is considered a positive sample. Yellow (between 0.9 and 1.1) is considered an equivocal sample.

Table [SEQ Table * ARABIC]: Hematocrit Effects

Sample ID	Liaison Result	EDTA Whole Blood			EDTA Plasma		
		Mean RLU	CV %	Theranos Index	Mean RLU	CV %	Theranos Index
M1	Not Tested	129	26	0.12	128	19	0.12
M2	Not Tested	124	22	0.11	93	28	0.09
M3	Not Tested	201	24	0.18	150	25	0.14
M4	Not Tested	151	20	0.14	177	13	0.16
M5	Not Tested	225	16	0.21	256	15	0.23
F1	Not Tested	152	23	0.14	181	27	0.17
F2	Not Tested	115	13	0.10	188	24	0.17
F3	Not Tested	108	20	0.10	148	26	0.14
F4	Not Tested	130	21	0.12	199	27	0.18
F5	Not Tested	257	24	0.23	169	24	0.15

Table [SEQ Table * ARABIC]: Anti-coagulant Effects Format

Sample ID	Liason Result	EDTA Plasma			Li Hep Plasma			Serum			Theranos Result
		Mean RLU	CV %	Theranos Index	Mean RLU	CV %	Theranos Index	Mean RLU	CV %	Theranos Index	
MEB1	Negative	179	26	0.17	149	23	0.14	158	31	0.15	Negative
MEB2	Negative	112	5	0.10	128	15	0.12	146	9	0.14	Negative
MEB3	Negative	154	28	0.14	171	10	0.16	293	28	0.27	Negative
MEB4	Negative	167	20	0.16	132	23	0.12	218	15	0.20	Negative
MEB5	Negative	140	24	0.13	172	30	0.16	160	26	0.15	Negative
FEB1	Negative	128	19	0.12	133	29	0.12	179	25	0.17	Negative
FEB2	Negative	141	16	0.13	152	26	0.14	171	19	0.16	Negative
FEB3	Positive	14086	18	13.18	11025	14	10.31	13328	24	12.47	Positive
FEB4	Negative	115	15	0.11	116	34	0.11	130	9	0.12	Negative

2.11 Effect of Interfering Matrixes

Icteric, Lipemic, and Hemolysed samples were tested and compared to the predicate method. All samples matched the Liaison result. Samples were run in triplicate with two tips per cartridge.

The Theranos index was calculated by using the established cutoff and the following equation:

RLU/1069=Theranos Index

Green (below 0.9 Index) is a considered a negative sample. Red (above 1.1 Index) is considered a positive sample. Yellow (between 0.9 and 1.1) is considered an equivocal sample.

Table [SEQ Table * ARABIC]: Interfering Matrixes

Sample ID	Liason Result	Mean RLU	CV %	Theranos Index	Theranos Result
L683	Positive	3546	28	3.32	Positive
L684	Negative	208	25	0.19	Negative
L685	Positive	12803	11	11.98	Positive
L687	Positive	6393	35	5.98	Positive
L689	Positive	22797	13	21.32	Positive
H03	Negative	408	12	0.38	Negative
H04	Positive	2851	15	2.67	Positive
H07	Negative	482	19	0.45	Negative
H08	Positive	14382	27	13.45	Positive
H09	Positive	3403	27	3.18	Positive
I26	Negative	135	22	0.13	Negative
I4	Negative	173	27	0.16	Negative
I9	Positive	1262	9	1.18	Positive
I302	Positive	27920	18	26.12	Positive
I525	Negative	188	18	0.18	Negative

2.12 Cross Reactivity

The assay was tested for potential cross reactivity from HAMA and RF samples. HAMA and RF samples that were run on the Theranos system correlated well with the Liaison results. Samples were run in triplicate with two tips per cartridge.

The Theranos index was calculated by using the established cutoff and the following equation:

RLU/1069=Theranos Index

Green (below 0.9 Index) is a considered a negative sample. Red (above 1.1 Index) is considered a positive sample. Yellow (between 0.9 and 1.1) is considered an equivocal sample.

Table [SEQ Table * ARABIC]: RF and HAMA

HAMA						
Sample ID	Liason Result	EuroImmunResult	Mean RLU	CV %	Theranos Index	Theranos Result
HA01	Negative	Negative	273	14	0.25	Negative
HA02	Negative	Negative	160	30	0.15	Negative
HA03	Negative	Negative	141	23	0.13	Negative
HA04	Negative	Negative	244	48	0.23	Negative
HA05	Positive	Positive	12805	24	11.98	Positive
HA06	Negative	Negative	218	20	0.20	Negative
HA07	Negative	Negative	178	32	0.17	Negative
HA08	Negative	Negative	215	15	0.20	Negative
HA09	Positive	Positive	5018	21	4.69	Positive
HA10	Positive	Negative	2671	30	2.50	Positive
HA11	Positive	Negative	1525	26	1.43	Positive
RF						
Sample ID	Liason Result	EuroImmunResult	Mean	CV %	Theranos Index	Theranos Result
RF01	Negative	Negative	223	21	0.21	Negative
RF02	Negative	Negative	187	39	0.17	Negative
RF03	Negative	Negative	167	20	0.16	Negative
RF04	Positive	Positive	5417	18	5.07	Positive
RF05	Negative	Negative	172	22	0.16	Negative
RF06	Positive	Positive	35056	13	32.79	Positive
RF07	Negative	Negative	302	13	0.28	Negative
RF08	Negative	Negative	143	14	0.13	Negative
RF09	Negative	Negative	154	14	0.14	Negative
RF10	Negative	Negative	169	19	0.16	Negative
RF11	Positive	Positive	3420	12	3.20	Positive
RF12	negative	Positive	432	21	0.40	Negative
RF13	Positive	Negative	531	27	0.50	Negative
RF14	Positive	Positive	5021	25	4.70	Positive
RF15	Positive	Positive	14845	28	13.89	Positive

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Table [SEQ Table * ARABIC]: Cross Reactive Samples

Sample ID	Liaison Result	Mean RLU	CV %	Theranos Index	Theranos Result
BRN	Negative	134	22	0.13	Negative
BRP	Positive	1761	15	1.65	Positive
Trep IgG	Positive	2609	31	2.44	Positive
CMV IgG	Negative	324	21	0.30	Negative
Rubella IgG	Negative	207	18	0.19	Negative
Toxo IgG	Negative	502	28	0.47	Negative
EBV IgG	Negative	883	13	0.83	Negative
VZV IgG	Negative	446	28	0.42	Negative
Ct/Ng	Negative	146	17	0.14	Negative
Toxo IgM	Equivocal	935	18	0.87	Negative
EBV IgM	Negative	1063	13	0.99	Equivocal
CMV IgM	Positive	2248	24	2.10	Positive
HPV IgG	Not Tested	5648	24	5.28	Positive
C. albicans	Negative	142	25	0.13	Negative

2.13   Reference method Clinical Correlation

The Theranos assay was tested using samples provided by BioReclamation, Zeptometrix, and SeraCare. The results were compared to the predicate method and a secondary kit. Samples were run in duplicate with two tips per cartridge.

The Theranos index was calculated by using the established cutoff and the following equation:

RLU/1069=Theranos Index

Green (below 0.9 Index) is a considered a negative sample. Red (above 1.1 Index) is considered a positive sample. Yellow (between 0.9 and 1.1) is considered an equivocal sample.

Table [SEQ Table * ARABIC]: Reference method Correlation

Liaison HSV1 Result	Sample ID	Liaison HSV2 Result	EuroImmun HSV2 Result	Theranos Index	Theranos Result
Negative	BR113	Positive	Positive	23.34	Positive
Negative	BR202	Positive	Positive	29.79	Positive
Negative	BR203	Positive	Positive	7.94	Positive
Negative	BR204	Positive	Positive	2.63	Positive
Negative	BR207	Positive	Positive	15.77	Positive
Negative	BR208	Positive	Positive	24.24	Positive
Negative	BR209	Positive	Positive	14.48	Positive
Negative	BR211	Positive	Positive	24.93	Positive
Negative	BR213	Equivocal	Negative	0.74	Negative
Negative	BR215	Positive	Positive	13.33	Positive
Negative	Z16	Positive	Equivocal	5.14	Positive
Negative	Z21	Positive	Positive	9.96	Positive
Negative	Z22	Positive	Equivocal	5.22	Positive
Negative	Z23	Positive	Equivocal	3.63	Positive
Negative	F03	Positive	Positive	13.34	Positive
Negative	SC03	Positive	Positive	10.56	Positive
Negative	SC04	Positive	Positive	36.49	Positive
Negative	SC05	Positive	Positive	14.44	Positive
Negative	SC11	Positive	Positive	13.15	Positive
Negative	SC15	Positive	Positive	4.80	Positive
Negative	MT02	Positive	Positive	18.50	Positive
Negative	MT06	Positive	Equivocal	2.82	Positive
Negative	MT07	Positive	Equivocal	0.78	Negative
Negative	MT08	Positive	Positive	23.58	Positive
Negative	MT13	Positive	Positive	17.13	Positive
Negative	MT16	Positive	Positive	3.54	Positive
Negative	MT17	Positive	Positive	22.53	Positive
Negative	MT24	Positive	Equivocal	3.33	Positive
Positive	BR108	Positive	Positive	17.76	Positive
Positive	BR110	Positive	Positive	10.53	Positive
Positive	BR118	Positive	Positive	21.22	Positive
Positive	BR201	Positive	Positive	14.83	Positive
Positive	BR206	Positive	Positive	18.70	Positive
Positive	BR210	Positive	Positive	8.29	Positive
Positive	BR212	Positive	Positive	17.80	Positive
Positive	BR214	Positive	Positive	8.72	Positive

|					
Positive	BR216	Positive	Positive	16.08	Positive
Positive	Z08	Positive	Positive	9.03	Positive
Positive	Z09	Positive	Negative	1.27	Positive
Positive	Z10	Positive	Equivocal	2.46	Positive
Positive	Z17	Positive	Positive	5.52	Positive
Positive	SC06	Positive	Positive	2.39	Positive
Positive	SC08	Positive	Positive	8.51	Positive
Positive	SC09	Positive	Positive	9.09	Positive
Positive	SC12	Positive	Negative	1.87	Positive
Positive	SC14	Positive	Positive	13.15	Positive
Positive	MT03	Positive	Positive	0.54	Negative
Positive	MT04	Positive	Positive	8.63	Positive
Positive	MT09	Positive	Positive	14.37	Positive
Positive	MT11	Positive	Positive	15.30	Positive
Positive	MT23	Positive	Negative	0.17	Negative
Positive	MT25	Positive	Equivocal	3.44	Positive
Positive	BR101	Negative	Negative	0.19	Negative
Positive	BR102	Negative	Negative	0.36	Negative
Positive	BR103	Negative	Negative	0.30	Negative
Positive	BR104	Negative	Negative	0.16	Negative
Positive	BR105	Negative	Negative	0.24	Negative
Positive	BR106	Negative	Negative	0.13	Negative
Positive	BR107	Negative	Negative	0.13	Negative
Positive	BR109	Negative	Equivocal	0.11	Negative
Positive	BR111	Negative	Positive	0.14	Negative
Positive	BR112	Negative	Negative	0.11	Negative
Positive	BR114	Negative	Negative	0.13	Negative
Positive	BR115	Negative	Negative	0.37	Negative
Positive	BR116	Negative	Negative	0.12	Negative
Positive	BR117	Negative	Equivocal	0.14	Negative
Positive	Z20	Negative	Negative	0.67	Negative
Positive	Z25	Negative	Negative	1.29	Positive
Positive	Z27	Negative	Negative	0.14	Negative
Positive	Z29	Negative	Negative	0.26	Negative
Positive	Z30	Negative	Negative	0.37	Negative
Positive	Z39	Negative	Negative	0.21	Negative

2.1 Stability over 12 weeks

The BioRad ToRCH negative and positive was used to test stability of the coated surface and the working solution of detection stored at 4°C at 0,1,2,4,8,and 12 weeks. After 12 weeks the reagents have shown to be stable.

Table [SEQ Table * ARABIC]: 12 Week Stability

	Week 0	Week 1	Week 2	Week 4	Week 7	Week 12	Average	CV
BioRad Negative	191	160	268	186	123	157	186	29
BioRad Positive	2391	2354	2502	2422	2358	2489	2405	3

Figure [SEQ Figure * ARABIC]: Reagent stability over 12 weeks

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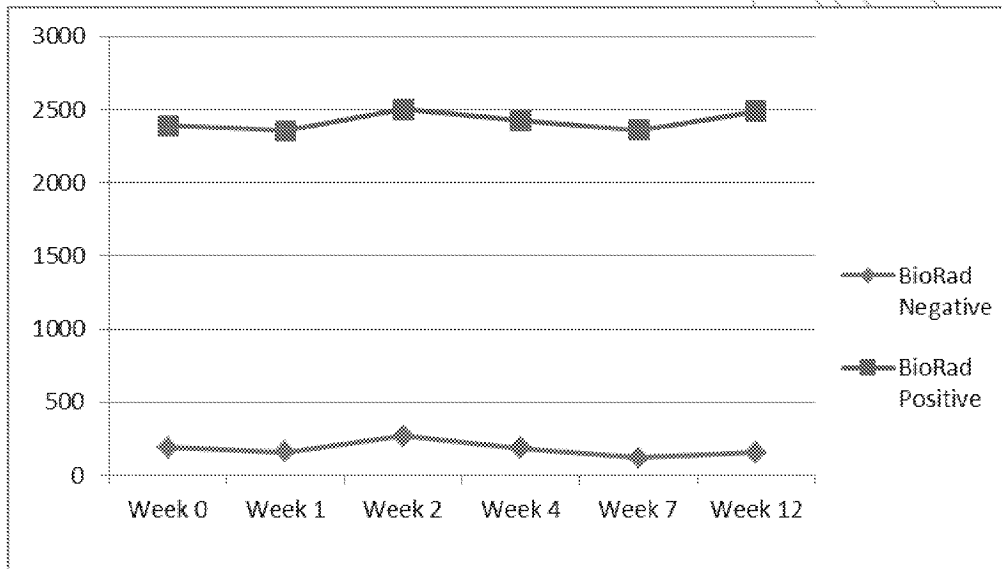
3   ASSAY SUMMARY

Table [SEQ Table * ARABIC]: Development Summary

Capture Antigen	HSV2 antigen @ 5 ug/mL
Wash Buffer	Tris Buffered Saline with Tween 20
Assay Buffer	Surmodics Assay Diluent (Protein Free)
Edison Protocol	25x_PSW_5-5-5
Detector Antibody	Novus clone 2C11 @ 25ng/mL
Detector Stabilizer	Theranos AP Conjugate Stabilizer
Sample Dilution	25x

4 CLINICAL EVALUATION

To further validate the assay, more normal and positive samples need to be tested. The cutoff value needs to be verified by screening more normal patients. At least 100 or more patients are needed for clinical evaluation.

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