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Parvovirus Human IgG Assay Feasibility Report

Date: 12 February 2013

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1 ASSAY INFORMATION

1.1 Assay Specifications

An enzyme linked immunosorbent assay (ELISA) was developed for the qualitative detection of human IgG antibodies to Parvovirus B19 virus in human serum, plasma (li-Heparin, EDTA) and whole blood (EDTA). Human B19 IgG Elisa intended used for preliminary diagnosis of recent or current infection. Blood test can determine if the person is susceptible, immune to infection or recently infected. The infections of Parvovirus usually go away on its own. This disease mainly effect grade school age children and spread through droplet (respiratory secretion). There is no significant symptom to detect initial Parvovirus infection; therefore testing woman of childbearing age to detect for the present of Parvovirus IgG is important. Parvovirus infection may lead to serious complication in pregnant woman because it can cause the baby to become anemic. Patient with immunocompromised or pregnant patient of severely anemic fetus might lead to life-threatening complication. Physician can also use this assay kit for diagnosis of fifth disease in children. Parvovirus B19 IgM detection is recommended to test in conjunction with detection of Parvovirus IgG to confirm the serological status of the patient.

This report describes the assay development and performance of the Human Parvovirus B19 IgG immunosorbent assay (ELISA) using Theranos Assay Systems.

Reference Assays

- Liaison-Biotrin Parvovirus B19 IgG Enzyme Immunoassay, cat# 519IGUS

1.1.1 Materials and Methods

The Human Parvovirus B19 IgG enzyme immunoassay is a direct antigen (Parvovirus B19 virus like particles-VLP) capture immunoassay for detection of Parvovirus IgG in patient serum, plasma (li-heparin, EDTA) and blood (EDTA). The Parvovirus IgG present in serum, plasma and blood will bind to Parvovirus capture antigen for 10 minutes followed by wash cycle to wash away unbound Parvovirus IgG and other non-specific immunoglobulin. After the wash step, detection antibodies mouse anti-human IgG alkaline phosphatase conjugate is added. Mouse anti-human IgG-AP recognized and bind to the present Parvovirus IgG-Parvovirus antigen complex. Incubation of this complex for 10 minutes, follow by wash cycle to wash away unbound detection antibodies. The whole complex mouse anti human IgG, Parvovirus IgG and Parvovirus antigen is detected by addition of Alkaline phosphatase substrate and incubates for 10 minutes. AP conjugate mouse anti human IgG activated by AP substrate result in enzyme activation of chemiluminescense is read in relative light units (RLU) on Theranos system.

Table 1: Materials

Name	Supplier	Catalog #
Parvovirus B19 VLP VP2 antigen	Diarect	48000
Mouse Anti-hIgG clone 2C11	Novus	NB100-2046
Parvovirus B19 IgG Enzyme Immunoassay	Liaison-Biotrin	519IGUS
Alkaline Phosphatase Labeling Kit (SH)	Dojindo	LK13-10
Theranos Substrate	Theranos	In House
Assay Diluent (Protein Free)	Pierce	37570
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

All experiments used three cartridges per sample with two tips per cartridge per condition unless otherwise specified.

2.1 Antigen Capture

Parvovirus B19 virus-like particles (VLP) capsid antigen VP2 and co-capsid VP1/VP2 antigen were tested to determine the best capture antigen. Human Parvovirus B19 is a human-pathogenic parvovirus consisting of a small non-enveloped particle with a single-stranded liner 5.6-kb DNA genome. The icosahedral capsid consists of two structure proteins, VP1 (53kDa) and VP2 (58kDa), which are identical with the exception of 227 amino acids at the animo-terminal end of the VP1-protein.

The Theranos tips were coated with antigen in Carbonate-Bicarbonate buffer at 5ug/ml. The assay was performed using Generic2-100x-PSW-10-10-10 protocol. Alkaline phosphatase conjugate mouse anti human IgG clone 2C-11 detection antibody was tested at 100ng/ml in Theranos 3% BSA assay buffer. Samples were tested in triplicate with two tips per cartridge.

Both capture antigens performed well resulting in high modulation compared to assay buffer (3% BSA) alone, no sample. Overall inter-cartridge percentage of CV was less than 25%. Theranos tips coated with Parvovirus B19 VLP VP2 resulted in lower RLU count for normal donors (parvovirus IgG negative, confirmed on the reference assay) and significant higher RLU count for Parvovirus positive donors. Therefore capture antigen VP2 was the lead capture antigen reagent for this assay development (Table 2). The modulation was calculated using the response of the samples compared to assay buffer matrix

Table 2: Antigen Capture Screen

	PV VLP VP2 (Cab3)			PV VLP VP1 VP2 (Cab4)		
	Inter Cartridges			Inter Cartridges		
Sample ID	Ave RLU	%CV	Modulation	Ave RLU	%CV	Modulation
Stanford Normal Donor 1	514	17.8	2	16235	14.5	59
Stanford Normal Donor 2	2851	26.5	9	4157	23.0	15
Stanford Normal Donor 7	41373	17.6	136	80333	20.3	292
Stanford Normal Donor 16	296	22.6	1	523	22.6	2
Stanford Normal Donor 17	3687	20.3	12	4218	25.1	15
Seracare Positive Titer 1	151491	25.5	498	374495	12.0	1361
Seracare Positive Titer 2	135841	20.4	446	372118	23.9	1352
Seracare Positive Titer 3	144055	31.2	473	294897	14.0	1072
Seracare Positive Titer 4	58668	26.5	193	103247	18.3	375
Seracare Positive Titer 5	269201	46.1	885	429442	19.8	1560
3% BSA	304	19.6	1	275	25.6	1

2.2 Capture Surface Titration

Parvovirus B19 VLP VP2 capture antigen was titrated to determine optimal surface concentration. Theranos tips coated with VP2 antigen at 10ug/ml, 5ug/ml, 2.5ug/ml and 1.25ug/ml were evaluated. Reader protocol was Generic2-100x-PSW-10-10-10. Novus clone 2C11 mouse anti human IgG-AP conjugated detection antibodies was used at 100ng/ml in Theranos 3% assay buffer. Samples were tested in triplicate with two tips per cartridge per samples.

Antigen concentration at 5ug/ml and 2.5ug/ml performed better than 10ug/ml and 1.25ug/ml tips capture for modulation and precision. For further experiments, VP2 antigen capture antigen at 2.5ug/ml in Carbonate-Biocarbonate buffer was selected. Seracare clinical samples with low titer samples were tested to ensure that the assay was able to detect low positive samples (Table 3 and Table 4).

Table 3: Capture Titration 10ug/ml and 5ug/ml B19 VLP VP2

	Capture Antigen = 10ug/ml			Capture Antigen = 5ug/ml		
	Inter Cartridges			Inter Cartridges		
Sample ID	Ave RLU	%CV	Modulation	Ave RLU	%CV	Modulation
Bloodbank donor samples (pooled 1,2,7,16,17)	38740	17.7	76	14725	13.1	47
Seracare Positive Plasma 3	579010	18.3	1143	166684	15.1	529
Seracare Positive Plasma 4	207796	9.9	410	73418	20.3	233
Seracare Mix Titer 4 (low positive)	88423	11.6	174	24525	21.9	78
Seracare Mix Titer 6 (low positive)	86606	13.4	171	46988	20.5	149
Seracare Mix Titer 8 (low positive)	43629	25.3	86	16536	20.9	52
Seracare Mix Titer 9 (low positive)	296731	6.3	586	84298	17.9	268
Seracare Mix Titer 21 (low positive)	822253	17.1	1623	35569	12.7	113
3% BSA	507	36.7	1	315	25.9	1

Table 4: Capture Titration 5ug/ml and 1.25ug/ml B19 VLP VP2

	Capture Antigen = 2.5ug/ml			Capture Antigen = 1.25ug/ml		
	Inter Cartridges			Inter Cartridges		
Sample ID	Ave RLU	%CV	Modulation	Ave RLU	%CV	Modulation
Bloodbank donor samples (pooled 1,2,7,16,17)	17142	33.8	54	10298	20.6	28
Seracare Positive Plasma 3	427465	23.5	1342	85188	16.9	229
Seracare Positive Plasma 4	123685	20.9	388	47584	20.0	128
Seracare Mix Titer 4 (low positive)	38284	22.4	120	13659	13.4	37
Seracare Mix Titer 6 (low positive)	53127	15.6	167	19887	14.8	53
Seracare Mix Titer 8 (low positive)	20108	17.9	63	12078	16.3	32
Seracare Mix Titer 9 (low positive)	142598	17.5	448	41907	18.9	113
Seracare Mix Titer 21 (low positive)	445680	5.3	1400	16188	25.4	44
3% BSA	318	23.0	1	372	20.0	1

2.3 Detection Antibody Stabilizer

To test the effect of detection antibody stabilizers, Novus clone 2C11 was diluted in Theranos AP conjugate Stabilizer, Surmodics Stabilzyme, and Sigma Biostab at a concentration 100 ng/mL. The assay was performed using the Generic2_100x-PSW-10-10-10 and capture antigen at 2.5ug/ml. The best performing diluent was Theranos AP Conjugate Stabilizer. Samples were run in triplicate with two tips per cartridge.

Stablizyme and Biostab Stablizyme did not yield acceptable modulation between parvovirus IgG positive and negative samples. With the Theranos Stabilizer the separation between normal donors and positive titer samples remain high (Table 5).

Table 5: AP Conjugate Stabilizer

	Theranos Stabilizer			Surmodics Stabilzyme			Sigma Biostab Stabilzyme		
	Inter Cartridges			Inter Cartridges			Inter Cartridges		
Sample ID	Ave RLU	%CV	Modulation	Ave RLU	%CV	Modulation	Ave RLU	%CV	Modulation
Stanford Normal Donor 2	318	25.8	1	245	28.6	1	421	37.1	1
Stanford Normal Donor 3	324	25.4	1	222	19.4	1	600	29.4	1
Stanford Normal Donor 5	371	27.2	1	254	24.1	1	533	28.4	1
Stanford Normal Donor 10	301	17.4	1	350	35.2	1	403	7.4	1
Stanford Normal Donor 24	212	11.1	1	242	16.3	1	541	25.2	1
Stanford Normal Donor 31	557	24.6	2	267	30.2	1	633	29.3	1
Stanford Normal Donor 49	271	27.3	1	341	25.3	1	398	43.2	1
Stanford Normal Donor 51	352	11.4	1	219	19.4	1	430	21.2	1
Seracare Mix Titer 2 (positive)	40276	17.6	125	6628	11.3	25	3736	14.8	7
Seracare Mix Titer 6 (low positive)	15310	29.9	48	2354	13.2	9	2640	10.6	5
Seracare Mix Titer 21 (low positive)	14520	54.6	45	2473	8.2	9	2031	22.8	4
Seracare Mix Titer 10 (negative)	1782	23.2	6	628	10.7	2	3768	18.5	7
3% BSA	302	20.4	1	539	22.6	2	467	38.1	1
SunnyLab Positive Plasma	155271	13.8	483	33688	6.6	127	161610	18.5	315

2.4 Sample Dilution Effect

To determine the optimal dilution factor for samples, 100x, 250x and 500x were tested. Reader protocol Generic2-100x-PSW-10-10-10 and Novus clone 2C11 mouse anti human IgG-AP conjugate at 100ng/ml in Theranos stabilizer were used in this assay.

100x sample dilution gave the best modulation between the parvovirus IgG positive and negative samples whereas for the higher dilutions modulation was greatly reduced (Table 6).

Table 6: Sample Dilution Effect

	Sample Dilution = 100x			Sample Dilution = 250x			Sample Dilution = 500x		
	Inter Cartridges			Inter Cartridges			Inter Cartridges		
Sample ID	Ave RLU	%CV	Modulation	Ave RLU	%CV	Modulation	Ave RLU	%CV	Modulation
Stanford Normal Donor 5	145	26.6	1	187	39.9	1	126	22.6	1
Stanford Normal Donor 10	220	22.5	1	149	14.8	1	128	39.3	1
Stanford Normal Donor 31	153	26.8	1	160	45.5	1	117	39.1	1
Stanford Normal Donor 48	166	25.6	1	118	21.0	1	152	34.3	1
Stanford Normal Donor 49	180	25.7	1	166	33.3	1	168	19.3	1
Stanford Normal Donor 50	135	31.5	1	139	22.4	1	155	22.1	1
Stanford Normal Donor 51	153	30.3	1	135	29.4	1	152	33.3	1
Stanford Normal Donor 53	153	21.9	1	115	39.1	1	122	26.7	1
Stanford Normal Donor 54	130	33.4	1	119	42.2	1	109	19.2	1
Stanford Normal Donor 55	175	38.2	1	159	42.2	1	114	16.3	1
Seracare Mix Titer 1 (positive)	12907	14.0	80	3109	56.9	21	2118	24.1	16
Seracare Mix Titer 19 (positive)	23136	2.5	144	4434	67.1	31	4399	36.2	33
Seracare Mix Titer 4 (low positive)	5508	12.2	34	1011	34.0	7	1039	18.2	8
Seracare Mix Titer 6 (low positive)	7128	17.2	44	2187	39.2	15	1369	32.3	10
Seracare Mix Titer 8 (low positive)	3607	10.2	22	858	33.0	6	454	50.6	3
Seracare Mix Titer 9 (low positive)	18904	18.9	117	6901	46.0	48	4590	15.8	34
Seracare Mix Titer 7 (negative)	1321	10.5	8	461	20.5	3	338	21.6	3
Seracare Mix Titer 11 (negative)	784	38.2	5	272	32.9	2	199	25.8	1
Seracare Mix Titer 17 (negative)	387	20.1	2	229	15.1	2	153	29.0	1
3% BSA	162	24.9	1	127	40.7	1	110	23.5	1
SunnyLab Positive Plasma	81589	11.8	506	18980	48.6	131	21134	16.4	157

2.5 Detector Antibody Titration

The anti-human IgG-AP conjugated was titrated at 50ng/ml, 25ng/ml and 12.5ng/ml to determine optimal concentration

Detection antibodies concentration at 25ng/ml was determined to be the optimal for the assay (Table 7)

Table 7: Detection Titration

	Detection Ab=50ng/ml			Detection Ab=25ng/ml			Detection Ab=12.5ng/ml		
	Inter Cartridges			Inter Cartridges			Inter Cartridges		
Sample ID	Ave RLU	%CV	Modulation	Ave RLU	%CV	Modulation	Ave RLU	%CV	Modulation
Stanford Normal Donor 5	265	36.7	1	145	26.6	1	113	26.7	1
Stanford Normal Donor 10	238	23.1	1	220	22.5	1	104	36.3	1
Stanford Normal Donor 31	243	29.1	1	153	26.8	1	122	24.7	1
Stanford Normal Donor 48	198	20.9	1	166	25.6	1	92	22.9	1
Stanford Normal Donor 49	252	26.1	1	180	25.7	1	166	56.3	1
Stanford Normal Donor 50	255	68.6	1	135	31.5	1	242	64.9	2
Stanford Normal Donor 51	245	28.1	1	153	30.3	1	97	9.9	1
Stanford Normal Donor 53	229	32.2	1	153	21.9	1	189	48.9	1
Stanford Normal Donor 54	224	30.9	1	130	33.4	1	98	24.8	1
Stanford Normal Donor 55	136	15.8	0	175	38.2	1	102	18.3	1
Seracare Mix Titer 1 (positive)	47733	15.9	152	12907	14.0	80	6339	48.1	48
Seracare Mix Titer 19 (positive)	34661	29.0	110	23136	2.5	144	11561	22.3	87
Seracare Mix Titer 4 (low positive)	8320	13.6	26	4002	7.4	25	844	62.9	6
Seracare Mix Titer 6 (low positive)	15594	37.1	50	7128	17.2	44	6001	37.0	45
Seracare Mix Titer 8 (low positive)	2924	19.5	9	3607	10.2	22	1563	41.0	12
Seracare Mix Titer 9 (low positive)	40396	16.3	129	18904	18.9	117	5246	20.1	40
Seracare Mix Titer 7 (negative)	2724	10.3	9	1321	10.5	8	615	33.8	5
Seracare Mix Titer 11 (negative)	1343	25.2	4	784	38.2	5	264	37.8	2
Seracare Mix Titer 17 (negative)	672	26.4	2	387	20.1	2	268	14.6	2
3% BSA	294	53.9	1	162	24.9	1	77	11.9	1
SunnyLab Positive Plasma	122449	11.5	390	81589	11.8	506	30535	30.4	230

2.6 Effect of Assay Diluent

Several assay buffers were tested to determine the optimal sample diluent for this assay. Theranos 3% BSA assay buffer, Theranos 3% assay buffer with 200ug/ml HBR, Pierce Protein-Free, Pierce Starting Block, Pierce Superblock and Surmodics Protein-Free assay buffer.

Pierce Protein-Free assay diluent gave lower background (low RLU) count for the parvovirus IgG negative samples, thus yielding a larger separation of positive and negative titer samples. Pierce SuperBlock assay diluent also yielded similar result as Pierce Protein-Free buffer. Pierce Protein-Free assay buffer also gave lower inter-cartridge % CV. During this step of assay optimization, additional clinical samples were tested with the Pierce Protein-Free to ensure good correlation with the reference assay results (Table 8 to Table 12).

The Antibody index was calculated using the following formula:

Antibody Index = Mean RLU (confirmed negative donors) + (10* Standard deviation (confirmed negative donors))

Table 8: Theranos 3% BSA assay buffer

3% BSA	Inter Cartridges			Theranos	Biotrin
Sample ID	Ave RLU	%CV	Modulation	Ab Index	Ab Index
Stanford Normal Donor 20	2321	11.7	0	0.01	0.34
Bioreclamation Normal Donor 58	1650	22.2	0	0.01	0.07
Bioreclamation Normal Donor 4	1828	16.7	0	0.01	0.20
Bioreclamation Normal Donor 3	3380	14.7	0	0.01	0.20
Bioreclamation Normal Donor 8	78852	11.7	5	0.26	0.10
Bioreclamation Normal Donor 12	20994	15.0	1	0.07	0.20
Seracare Positive Titer 3	115797	20.6	7	0.39	7.36
Seracare Positive Titer 4	48895	13.4	3	0.16	6.38
WHO 10IU/ML	172164	18.2	10	0.58	
Seracare Mix Titer 10	902	17.3	0	0.00	0.30
Seracare Mix Titer 12	1131	18.5	0	0.00	0.30
Seracare Mix Titer 15	961	11.9	0	0.00	0.10
Seracare Mix Titer 4	4213	17.0	0	0.01	2.10
Seracare Mix Titer 6	9124	11.8	1	0.03	1.40
Seracare Mix Titer 21	7917	24.6	0	0.03	2.10

Table 9: Theranos 3% BSA assay buffer with Pierce Protein-Free, Surmodics Protein-Free assay buffers

	3% BSA AND Pierce Protein-Free				3% BSA AND Surmodics Protein-Free				
	Inter Cartridges			Theranos	Inter Cartridges			Theranos	Biotrin
Sample ID	Ave RLU	%CV	Modulation	Ab Index 10x	Ave RLU	%CV	Modulation	Ab Index 10x	Ab Index
Stanford Normal Donor 20	928	28.6	1	0.16	364	38.3	1	0.08	0.34
Bioreclamation Normal Donor 58	793	63.7	1	0.13	718	44.2	1	0.15	0.07
Bioreclamation Normal Donor Donor 4	1723	22.3	2	0.29	521	22.2	1	0.11	0.20
Bioreclamation Normal Donor 3	279	37.6	0	0.05	516	38.2	1	0.11	0.20
Bioreclamation Normal Donor 8	581	48.9	1	0.10	1321	29.5	2	0.29	0.10
Bioreclamation Normal Donor 12	284	42.0	0	0.05	646	19.6	1	0.14	0.20
Seracare Positive Titer 3	25239	29.3	36	4.24	50653	6.8	74	10.93	7.36
Seracare Positive Titer 4	26207	23.7	37	4.40	7435	34.7	11	1.60	6.38
WHO 10IU/ML	108250	17.0	155	18.17	32945	19.7	48	7.11	

Table 10: Theranos 3% BSA with Pierce Super Block, 200ug/ml HBR and Pierce Starting Block assay buffers

	3% BSA and Pierce Super Block				3% BSA and HBR-1 in 3% BSA				Pierce Starting Block				
	Inter Cartridges			Theranos	Inter Cartridges			Theranos	Inter Cartridges			Theranos	Biotrin
Sample ID	Ave RLU	%CV	Modulation	Ab Index 10x	Ave RLU	%CV	Modulation	Ab Index 10x	Ave RLU	%CV	Modulation	Ab Index 10x	Ab Index
Stanford Normal Donor 20	1615	23.0	0	0.02	992	11.8	0	0.00	1385	56.8	0	0.01	0.34
Bioreclamation Normal Donor 58	1747	19.2	0	0.02	1707	61.1	0	0.01	1584	34.9	0	0.01	0.07
Bioreclamation Normal Donor Donor 4	1286	36.5	0	0.01	1044	47.2	0	0.00	722	77.1	0	0.00	0.20
Bioreclamation Normal Donor 3	1386	19.5	0	0.01	3801	20.1	0	0.01	2828	41.7	0	0.02	0.20
Bioreclamation Normal Donor 8	27982	18.0	5	0.27	66842	15.8	4	0.24	42285	24.5	4	0.25	0.10
Bioreclamation Normal Donor 12	3329	27.6	1	0.03	13764	36.1	1	0.05	18318	11.7	2	0.11	0.20
Seracare Positive Titer 3	38790	26.7	7	0.38	89846	33.0	5	0.32	23566	24.1	2	0.14	7.36
Seracare Positive Titer 4	13312	101.5	2	0.13	68959	27.9	4	0.25	42745	74.5	4	0.26	6.38
WHO 10IU/ML	14650	27.1	3	0.14	21835	21.8	1	0.08	100354	26.2	9	0.60	

Table 11: Pierce Protein-Free and Surmodics Protein-Free assay buffers

	Pierce Protein Free Only				Surmodics Protein-Free Only				
	Inter Cartridges			Theranos	Inter Cartridges			Theranos	Biotrin
Sample ID	Ave RLU	%CV	Modulation	Ab Index 10x	Ave RLU	%CV	Modulation	Ab Index 10x	Ab Index
Stanford Normal Donor 20	114	6.9	1	0.29	207	24.0	1	0.10	0.34
Bioreclamation Normal Donor 58	133	19.5	1	0.33	101	17.9	0	0.05	0.07
Bioreclamation Normal Donor 4	114	9.2	1	0.29	99	13.1	0	0.05	0.20
Bioreclamation Normal Donor 3	117	24.0	1	0.30	600	22.5	3	0.28	0.20
Bioreclamation Normal Donor 8	129	29.3	1	0.32	194	36.9	1	0.09	0.10
Bioreclamation Normal Donor 12	146	19.8	1	0.37	129	19.2	1	0.06	0.20
Seracare Positive Titer 3	18189	19.6	144	45.77	35100	28.1	153	16.12	7.36
Seracare Positive Titer 4	10138	21.6	80	25.51	19111	21.1	83	8.78	6.38
WHO 10IU/ML	44238	19.1	349	111.31	62246	17.6	271	28.59	
Seracare Mix Titer 10	288	18.9	2	0.72	320	22.9	1	0.15	0.30
Seracare Mix Titer 12	367	27.5	3	0.92	311	29.8	1	0.14	0.30
Seracare Mix Titer 15	104	26.4	1	0.26	171	10.7	1	0.08	0.10
Seracare Mix Titer 4	1917	34.4	15	4.82	2158	23.2	9	0.99	2.10
Seracare Mix Titer 6	7463	21.2	59	18.78	5369	25.4	23	2.47	1.40
Seracare Mix Titer 21	5383	16.4	42	13.54	5393	15.0	23	2.48	2.10
Seracare Positive Titer 1	17778	29.6	140	44.73	11141	17.5	49	5.12	5.10
Seracare Positive Titer 2	12248	14.3	97	30.82	3863	27.0	17	1.77	4.60
Seracare Positive Titer 3	10229	15.4	81	25.74	6591	21.6	29	3.03	5.10
Seracare Positive Titer 5	31664	25.3	250	79.67	9259	25.0	40	4.25	5.60
Seracare Positive Titer 8	2412	19.5	19	6.07	1100	26.2	5	0.51	2.20
Seracare Positive Titer 9	30426	14.2	240	76.56	17342	25.6	76	7.97	2.60

Table 12: Super Block assay buffers

	Super Block Only				
	Inter Cartridges			Theranos	Biotrin
Sample ID	Ave RLU	%CV	Modulation	Ab Index 10x	Ab Index
Stanford Normal Donor 2	318	25.3	2	0.32	0.40
Stanford Normal Donor 3	217	19.2	1	0.22	0.24
Stanford Normal Donor 4	232	17.3	1	0.23	0.19
Stanford Normal Donor 20	107	24.4	1	0.11	0.34
Bioreclamation Normal Donor 58	128	21.2	1	0.13	0.07
Bioreclamation Normal Donor Donor 4	159	22.0	1	0.16	0.20
Bioreclamation Normal Donor 3	235	24.7	1	0.24	0.20
Bioreclamation Normal Donor 8	250	29.0	1	0.25	0.10
Bioreclamation Normal Donor 12	129	28.8	1	0.13	0.20
Seracare Positive Titer 3	24354	35.0	127	24.48	7.36
Seracare Positive Titer 4	9415	15.2	49	9.46	6.38
WHO 10IU/ML	63817	32.1	332	64.13	
Seracare Mix Titer 10	490	26.9	3	0.49	0.30
Seracare Mix Titer 12	748	21.2	4	0.75	0.30
Seracare Mix Titer 15	742	18.4	4	0.75	0.10
Seracare Mix Titer 4	3109	21.5	16	3.12	2.10
Seracare Mix Titer 6	6018	17.7	31	6.05	1.40
Seracare Mix Titer 21	3433	18.5	18	3.45	2.10
Seracare Positive Titer 1	22150	27.8	115	22.26	5.10
Seracare Positive Titer 2	23685	27.6	123	23.80	4.60
Seracare Positive Titer 5	41547	19.5	216	41.75	5.10
Seracare Positive Titer 7	93917	11.9	489	94.38	5.60
Seracare Positive Titer 8	41109	20.7	214	41.31	2.20
Seracare Positive Titer 9	17978	24.1	94	18.07	2.60

2.7 Effect of Assay Incubation time

To determine the optimal assay incubation time, three assay protocols were tested: Generic2-100x-PSW-10-10-10, Generic2-100x-PSW-5-5-5 and Generic2-100x-PSW-2-2-1. In all three assay protocols, samples were diluted at 100x in Pierce Protein Free assay buffer. All protocols included post sample wash to reduce non-specific binding.

Protocol Generic2-100x-PSW-10-10-10 was finalized due to the best modulation seen between the positive and negative samples (Table 13)

Table 13: Effect of Assay Incubation time

	Protocol Generic2-100x-PSW 10-10-10			Protocol Generic2-100x-PSW 5-5-5			Protocol Generic2-100x-PSW-2-2-1		
	Inter Cartridges			Inter Cartridges			Inter Cartridges		
Sample ID	Ave RLU	%CV	Modulation	Ave RLU	%CV	Modulation	Ave RLU	%CV	Modulation
Stanford Normal Donor 2	389	9.9	1	2011	27.3	1	922	31.9	1
Stanford Normal Donor 3	420	21.4	1	2255	14.4	1	1504	11.8	1
Stanford Normal Donor 4	392	11.4	1	1989	24.3	1	1468	14.1	1
Stanford Normal Donor 5	395	23.9	1	1977	24.7	1	429	19.5	0
Stanford Normal Donor 10	417	18.3	1	2912	19.3	1	1348	21.7	1
Stanford Normal Donor 13	336	22.1	1	1357	23.9	1	634	18.4	1
Seracare Positive Titer 1	16904	10.9	43	13556	21.1	6	3508	9.4	3
Seracare Positive Titer 2	29244	11.3	74	18740	21.8	9	3533	21.9	3
Seracare Positive Titer 3	26092	11.1	66	19586	14.8	9	3491	13.7	3
Seracare Mix Titer 4 (low positive)	3200	12.4	8	4221	17.5	2	1700	29.3	2
Seracare Mix Titer 6 (low positive)	8879	14.0	23	3964	23.5	2	4550	19.3	4
Seracare Mix Titer 8 (low positive)	1387	20.3	4	4425	20.7	2	1342	111.9	1

2.8 Antibody Index-Assay CutOff Value and Clinical Correlation

The presence or absent of Parvovirus B19 IgG was determined by antibody index.

Antibody Index = mean RLU/COV

To determine the COV:

1 30 negative samples from normal donors and 30 positive samples from Seracare B19 human Parvovirus Positive titer panel were confirmed by testing on reference assay.
2 Same set of positive and negative samples were tested again on Theranos assay using the optimized assay condition.

3 The assay CutOff Value for this assay was based on the clinical correlation of both negative samples and positive samples to the reference assay antibody index value and Theranos assay systems.

4 Calculation for COV for this assay:

COV= mean confirmed negative donors RLU + (10*Standard Deviation confirmed negative donors RLU)

Table 14: COV Calculation

<0.90 Negative ≤1.0 - ≥0.90 Equivocal >1.10 Positive	
Overall Average	211
SD	213
Mean + 10SD	2343

The Parovirus positive and negative titer samples correlated well with the results from reference assay (Table 15 and Table 16)

Table 15: Clinical correlation normal donors-Negative Titer

		Pavovirus Normal Donors			
		Inter Cartridges		Theranos	Biotrin
Sample #	Sample ID	Ave RLU	%CV	Ab Index 10X	Ab Index
1	Stanford Normal Donor 2	128	18	0.06	0.40
2	Stanford Normal Donor 3	191	22	0.08	0.24
3	Stanford Normal Donor 4	326	15	0.14	0.19
4	Stanford Normal Donor 5	257	20	0.11	0.23
5	Stanford Normal Donor 10	196	18	0.09	0.08
6	Stanford Normal Donor 13	122	9	0.05	0.21
7	Stanford Normal Donor 20	147	11	0.06	0.34
8	Stanford Normal Donor 21	122	13	0.05	0.29
9	Stanford Normal Donor 24	77	18	0.03	0.21
10	Stanford Normal Donor 49	142	11	0.06	0.50
11	Bioreclamation Normal Donor	138	15	0.06	0.05
12	Bioreclamation Normal Donor	144	22	0.06	0.07
13	Bioreclamation Normal Donor	221	20	0.10	0.06
14	Bioreclamation Normal Donor	101	6	0.04	0.13
15	Bioreclamation Normal Donor	94	16	0.04	0.06
16	Bioreclamation Normal Donor	255	18	0.11	0.06
17	Bioreclamation Normal Donor	301	17	0.13	0.07
18	Bioreclamation Normal Donor	208	25	0.09	0.06
19	Bioreclamation Normal Donor	153	17	0.07	0.06
20	Bioreclamation Normal Donor	216	18	0.09	0.06
21	Bioreclamation Normal Donor	213	21	0.09	0.20
22	Bioreclamation Normal Donor	315	16	0.14	0.20
23	Bioreclamation Normal Donor	158	25	0.07	0.20
24	Bioreclamation Normal Donor	154	20	0.07	0.20
25	Bioreclamation Normal Donor	1282	16	0.56	0.82
26	Bioreclamation Normal Donor	102	23	0.04	0.10
27	Bioreclamation Normal Donor	168	17	0.07	0.10
28	Bioreclamation Normal Donor	192	8	0.08	0.20
29	Bioreclamation Normal Donor	104	23	0.05	0.20
30	Bioreclamation Normal Donor	92	20	0.04	0.20

Table 16: Clinical correlation Seracare Positive Titer

		Pavovirus B19 Positive Donors			
		Inter Cartridges		Theranos	Biotrin
Sample #	Sample ID	Ave RLU	%CV	Ab Index 10X	Ab Index
1	Seracare Positive Titer 1	31383	12	13.67	7.52
2	Seracare Positive Titer 2	43388	14	18.90	7.27
3	Seracare Positive Titer 3	26851	19	11.70	7.36
4	Seracare Positive Titer 4	12430	18	5.41	6.38
5	Seracare Positive Titer 5	56796	24	24.74	7.28
6	Seracare Positive Titer 6	51658	19	22.50	7.01
7	Seracare Positive Titer 7	88529	15	38.57	5.23
8	Seracare Positive Titer 8	31634	21	13.78	6.21
9	Seracare Positive Titer 9	22517	17	9.81	4.56
10	Seracare Positive Titer 10	15221	26	6.63	6.27
11	Seracare Positive Titer 11	53161	21	23.16	6.65
12	Seracare Positive Titer 12	19963	25	8.70	5.09
13	Seracare Positive Titer 13	75049	13	32.70	5.92
14	Seracare Positive Titer 14	52442	20	22.85	6.50
15	Seracare Positive Titer 15	108177	6	47.13	6.89
16	Seracare Positive Titer 16	42529	21	18.53	6.04
17	Seracare Positive Titer 17	75966	16	33.09	7.22
18	Seracare Positive Titer 18	97569	13	42.51	6.60
19	Seracare Positive Titer 19	16431	16	7.16	5.80
20	Seracare Mix Titer 1	14844	25	6.47	5.10
21	Seracare Mix Titer 2	8411	18	3.66	4.60
22	Seracare Mix Titer 3	9534	16	4.15	5.10
23	Seracare Mix Titer 4	2920	24	1.27	2.10
24	Seracare Mix Titer 6	5005	20	2.18	1.40
25	Seracare Mix Titer 8	3755	13	1.64	2.20
26	Seracare Mix Titer 9	11917	21	5.19	2.60
27	Seracare Mix Titer 21	5569	12	2.43	2.10
28	Seracare Mix Titer 5	13348	18	5.82	5.60
29	Seracare Mix Titer 18	15502	18	6.75	4.80
30	Seracare Mix Titer 19	17819	17	7.76	5.40

2.9 Assay Specificity: RF, HAMA

The assay specificity was tested for cross reactivity with RF and HAMA samples using finalized condition.

There were no cross reactivity between Parvovirus B19 VLP VP2 capture antigen, detection antibody-towards these sample types. Theranos Parvovirus IgG assay correlated well with reference assay results (Table 17).

Table 17: Assay Specificity: RF, HAMA

	Inter Cartridges		Theranos	Biotrin		Inter Cartridges		Theranos	Biotrin
Sample ID	Ave RLU	%CV	Ab Index 10X	Ab Index	Sample ID	Ave RLU	%CV	Ab Index 10X	Ab Index
RF+1 ProMedDx 11672674	203	21	0.09	0.58	HAMA+1 ProMedDx 10989656	235	14	0.10	0.42
RF+2 ProMedDx 11673451	166	20	0.07	0.27	HAMA+2 ProMedDx 10538673	146	21	0.06	0.13
RF+3 ProMedDx 11672760	255	21	0.11	0.67	HAMA+3 ProMedDx 10538677	99	9	0.04	0.13
RF+4 ProMedDx 11745823	112	11	0.05	0.13	HAMA+4 ProMedDx 10580267	147	20	0.06	0.14
RF+5 ProMedDx 11745846	132	22	0.06	0.14	HAMA+5 ProMedDx 10538690	178	23	0.08	0.13

2.10 Anti-Coagulant Effect

To test for effect of different anti-coagulants, matching samples of serum, EDTA plasma, Li-Heparin plasma were collected from 5 male donors, 5 female donors of serum using finalized assay condition.

Anti-coagulants showed no effect on the outcome of the assay results. Serum, EDTA plasma or Li-Heparin anti-coagulants yielded similar results. Antibody index of these samples also correlated well with reference assay (Table 18)

Table 18: Anti-Coagulants Effect and Antiboy Index correlation compare to reference assay

	Serum			Li-Heperin Plasma			EDTA Plasma			
	Inter Cartridges		Theranos	Inter Cartridges		Theranos	Inter Cartridges		Theranos	Biotrin
Sample ID	Ave RLU	%CV	Ab Index 10X	Ave RLU	%CV	Ab Index 10X	Ave RLU	%CV	Ab Index 10X	Ab Index
Male Donor 1	4527	23	1.97	8399	16	3.66	5201	16	2.27	2.23
Male Donor 2	524	25	0.23	243	20	0.11	246	20	0.11	0.36
Male Donor 3	555	21	0.24	230	25	0.10	308	9	0.13	0.35
Male Donor 4	27885	13	12.15	16224	22	7.07	39947	23	17.40	5.95
Male Donor 5	323	22	0.14	307	22	0.13	213	13	0.09	0.25
Female Donor 1	84813	15	36.95	140186	14	61.07	125235	14	54.56	7.17
Female Donor 2	3432	18	1.50	6690	22	2.91	13646	22	5.94	2.64
Female Donor 3	387	14	0.17	333	17	0.14	411	20	0.18	0.32
Female Donor 4	41346	20	18.01	32518	13	14.17	36932	23	16.09	6.73
Female Donor 5	281	14	0.12	379	21	0.17	617	4	0.27	0.35

2.11 Hematocrit Effect

The hematocrit effect was determined by collecting whole blood EDTA anticoagulant from 5 male and 5 female donors. The whole blood sample from each patient was split into two tubes. First tube was added directly whole blood sample into cartridge. Second tube, spin down to collect plasma. This EDTA plasma then added onto second set of cartridges to compare result from whole blood assay result. Assay finalized condition was used in this experiment.

There was no hematocrit effect when assay sample with whole blood or serum. Normal range slope equal to 1.0-2.0. All samples from 10 donors correlate well with reference assay results (Table 19 and Table 20)

Table 19: Hematocrit Effect Slope

EDTA Whole Blood/EDTA Plasma			
		Ave RLU	%CV
male 1	Whole Blood	4033	16
male 2	Whole Blood	2060	9
male 3	Whole Blood	290	24
male 4	Whole Blood	241	16
male 5	Whole Blood	15728	22
female 1	Whole Blood	1988	25
female 2	Whole Blood	424	21
female 3	Whole Blood	261	16
female 4	Whole Blood	1247	8
female 5	Whole Blood	19307	25
male 1	Plasma	12214	7
male 2	Plasma	3519	9
male 3	Plasma	297	24
male 4	Plasma	272	22
male 5	Plasma	15626	24
female 1	Plasma	4563	18
female 2	Plasma	268	23
female 3	Plasma	253	14
female 4	Plasma	2260	15
female 5	Plasma	28352	18

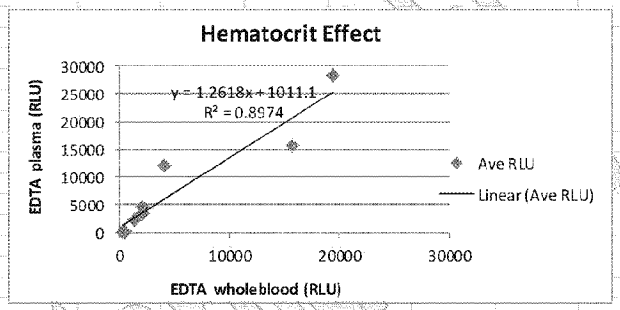

Table 20: Comparison of EDTA plasma and Whole Blood EDTA from same patients

	EDTA Plasma			Whole Blood EDTA			
	Inter Cartridges		Theranos	Inter Cartridges		Theranos	Biotrin
Sample ID	Ave RLU	%CV	Ab Index 10X	Ave RLU	%CV	Ab Index 10X	Ab Index
Male 1	12214	16	5.32	4033	7	1.76	5.30
Male 2	3519	9	1.53	2060	9	0.90	3.39
Male 3	297	24	0.13	290	24	0.13	0.34
Male 4	272	16	0.12	241	22	0.10	0.93
Male 5	15626	22	6.81	15728	24	6.85	6.07
Female 1	4563	25	1.99	1988	18	0.87	4.93
Female 2	268	21	0.12	424	23	0.18	0.45
Female 3	253	16	0.11	261	14	0.11	0.47
Female 4	2524	24	1.10	1247	15	0.54	2.66
Female 5	28352	25	12.35	19307	18	8.41	7.22

2.12 Cross Reactivity: Other Common Diseases

According to reference assay and literature about 80% of patients samples may cross react with antibodies from other common disease including Measles, Rubella, CMV, Mumps, HSV, Toxoplasma and Lyme disease. These cross reactants were evaluated in this experiment using assay finalized condition.

Assay results correlated well with the reference data (Table 21)

Table 21: Cross Reactivity

	Inter Cartridges		Theranos	Biotrin
Sample ID	Ave RLU	%CV	Ab Index 10x	Ab Index
Rubella IgG	12885	22	5.61	3.36
Seracare 140				
lot#12366				
Measles, Mumps, Rubella	2973	21	1.30	2.47
Seracare 40				
lot3 122049				
HSV IgG	637	19	0.28	0.85
Seracare 150				
lot#12247				
CMV IgG	8969	14	3.91	4.04
Seracare 145				
lot# 122727				
EBV IgG	15012	24	6.54	5.37
Seracare 30				
lot# 123537				
Toxoplasma IgG	7174	13	3.13	3.42
Seracare 135				
lot# 122955				
Borrella Burgdorferi IgG	5280	18	2.30	3.77
Seracare 130				
lot# 123789				

2.13 Matrix Effect

The effect of icteric, lypemic and hemolysed plasma samples on the assay was determined by testing 5 samples of each type using assay finalized condition. The result from this testing was compared to reference test. Note: The reference assay recommended not including icteric, lypemic and hemolysed samples for the kit.

The result from Theranos assay system correlated well with reference assay results (Table 22)

Table 22: Effect of Interfering Assay Matrix

	Inter Cartridges		Theranos	Biotrin
Sample ID	Ave RLU	%CV	Ab Index 10X	Ab Index
ProMedDx Icteric 4	5685	19	2.48	2.55
ProMedDx Icteric 5	458	16	0.20	0.05
ProMedDx Icteric 6	41806	19	18.21	6.28
ProMedDx Icteric 7	8723	13	3.80	3.53
ProMedDx Icteric 20	19122	7	8.33	4.99
Zeptometrix Hemolyzed 3	630	20	0.27	0.05
Zeptometrix Hemolyzed 4	5632	10	2.45	1.98
Zeptometrix Hemolyzed 5	996	13	0.43	0.05
Zeptometrix Hemolyzed 6	1069	23	0.47	0.06
Zeptometrix Hemolyzed 8	27499	17	11.98	6.17
ProMedDx Lypemic 7	4870	15	2.12	2.86
ProMedDx Lypemic 8	1681	13	0.73	0.76
ProMedDx Lypemic 9	1441	8	0.63	0.87
ProMedDx Lypemic 10	2123	19	0.92	1.17
ProMedDx Lypemic 11	26605	23	11.59	6.40

3. ASSSAY SUMMARY

Table 23: Development Summary

Capture Antigen	Diarect B19 VLP VP2-cat# 48000 @ 2.5ug/ml
Wash Buffer	Tris Buffered Saline with Tween 20
Assay Buffer	Pierce Protein-Free cat# 37570
Edison Protocol	Generic2-100x-10-10-10
Detector Antibody	Novus clone 2C11-cat# NB100-2046 @ 25ng/mL
Detector Stabilizer	Theranos AP Conjugate Stabilizer
Sample Dilution	100X

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 additional normal patients. At least 100 or more patients are needed for clinical evaluation.

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