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Trypansoma cruzi IgG Antibody Assay Development Report

September 26, 2012

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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"]

An enzyme linked immunosorbent assay (ELISA) was developed for the qualitative detection of Chagas' disease. Chagas' disease occurs mostly in Central and South America and is caused by the unicellular parasite Trypanosoma cruzi. Trypanosoma cruzi transmission to humans occurs through the bug bites of the family reduviidae. Chagas' disease is characterized by fever, diarrhea, grips, swollen lymp nodes and heart failure. This report describes the assay development and performance of the Theranos Chagas ELISA as aid to diagnosing Chagas' disease.

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

The following assay was used as a predicament method:

Chagas (Trypanosoma cruzi) IgG ELISA, IBL, Cat. RE58691)

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

The Theranos Chagas ELISA is a qualitative ELISA with an antigen surface. Human anti-Trypanosoma cruzi in serum or plasma bind specifically to the capture antigen for 10 minutes followed by a wash cycle. After washing, human anti-Trypanosoma cruzi IgG was detected using an AP labeled mouse monoclonal antibody to human IgG. After incubation with the detector antibody for10 minutes, another wash cycle was performed, and the alkaline phosphatase substrate added. The resulting chemiluminescence was read in relative light units (RLU) on the Theranos system.

Table [SEQ Table * ARABIC]: Materials

Item	Description	Vendor	Cat. #
Capture	[HYPERLINK "http://www.mybiosource.com/datasheet.php?products_id=537436" \o "Trypanosoma cruzi native protein"]	MyBiosource	MBS537
Detection Antibody	Mouse anti-human IgG clone 2C11	Novus	NB100-2(
Biorad Control	Chagas Single Level Control	Biorad	125
Seracare Control	Chagas Single Level Control	SeraCare	A190-50

1.1.3 Labeling of Detector Antibody

The mouse anti-human IgG clone 2C11 was labeled with alkaline phosphatase according to kit instructions (Dojindo, LK13-10).

1.1.4 Preparation of Assay Buffer

The assay buffer was prepared by dissolving 1 packet of TBS into water and adding 10 mL of 10% azide, and 30 g of BSA to a final volume of 1000mL. The final composition of the assay buffer is 3% BSA, 50mM Tris, 138mM NaCl, 2.7mM KCl pH 8.0 in water. The assay buffer was filtered before use.

1.1.5 Preparation of Theranos AP Stabilizer

The conjugate diluent was prepared by adding $Zn^{2+}Cl$ and $Mg^{2+}Cl$ to the assay buffer to final concentrations of 0.1mM Zn^{2+} and 5mM Mg^{2+}.

1.1.6 Preparation of Calibrators

The Chagas calibrators from Biorad and Seracare come ready to use.

2. ASSAY DEVELOPMENT

2.1 Surface Antigen Screen

Several antigen sources were tested to determine the best capture surface on the Theranos system. The protocol used was Generic2_5X_PSW and the detection antibody (Novus 2C11) was used at 100 ng/mL. The native Chagas antigen gave the highest modulation and was further used for assay development.

Table 2: Surface Antigen Screen

Antigen	Sample	AVG RLU	CV	Modulation
Composite Antigen	Biorad Chagas	26834	23%	4.7
Composite Antigen	Seracare Chagas	31396	3%	5.5
Composite Antigen	Biorad Syphilis	4713	12%	0.8
Composite Antigen	Human Serum	5675	30%	1.0
MACH Antigen	Biorad Chagas	102677	12%	0.3
MACH Antigen	Biorad Syphilis Level 3	312547	9%	0.9
MACH Antigen	Human Serum	316367	22%	1.0
Native Antigen	Biorad Chagas	127797	28%	11.3
Native Antigen	Biorad Syphillis Level 3	145461	11%	12.9
Native Antigen	Human Serum	11257	4%	1.0

2.2 Capture Surface Titration

The optimum concentration of Chagas native protein for surface coating was determined by comparing the assay performance at 1 ug/mL, 5 ug/mL, and 10 ug/mL. The modulation and assay precision was good at 10 ug/mL. The protocol used was Generic2_5X_PSW and the detection antibody was from Novus clone 2C11 at 100 ng/mL. Detection antibody clone JDC-10 (Southern Biotech) did not perform well.

Table 3: Capture Titration (Novus 2C11)

Chagas Native Protein	Sample	AVG RLU	CV	Modulation
1 ug/mL	Biorad Chagas	127797	28%	11.4
1 ug/mL	Biorad Syphillis Level 3	145461	11%	12.9
1 ug/mL	Human Serum	11257	4%	1.0
5 ug/mL	Biorad Chagas	795899	4%	34.3
5 ug/mL	Biorad Syphillis Level 3	164080	22%	7.1
5 ug/mL	Human Serum	23216	33%	1.0
10 ug/mL	Biorad Chagas	1269585	8%	33.9
10 ug/mL	Biorad Syphillis Level 3	172103	3%	4.6
10 ug/mL	Human Serum	37441	23%	1.0

Table 4: Capture Titration (Southern Biotech JDC-10)

Chagas Native Protein	Sample	AVG RLU	CV	Modulation
1 ug/mL	Biorad Chagas	586236	9%	3.0
1 ug/mL	Biorad Syphillis Level 3	695554	24%	3.6
1 ug/mL	Human Serum	195815	n/a	1.0
5 ug/mL	Biorad Chagas	2024896	5%	18.1
5 ug/mL	Biorad Syphillis Level 3	718355	11%	6.4
5 ug/mL	Human Serum	112112	18%	1.0
10 ug/mL	Biorad Chagas	2530722	6%	10.9
10 ug/mL	Biorad Syphillis Level 3	825952	3%	3.6
10 ug/mL	Human Serum	231932	6%	1.0

2.3 Detection Antibody Titration

The optimum detection antibody (Novus 2C11) concentration was determined by comparing the assay performance at 0.8 ng/mL, 4 ng/mL, 20 ng/mL, and 100 ng/mL. The modulation and assay precision was good at 4 ng/mL. The protocol used was Generic2_5X_PSW.

Table 5: Detection Antibody Titration

Dab	Sample	AVG RLU	CV	Modulation
100 ng/mL	Biorad Chagas	987099	14%	26.7
100 ng/mL	Biorad Syphilis Level 3	136535	15%	3.7
100 ng/mL	Human Serum	36949	20%	1.0
20 ng/mL	Biorad Chagas	273697	7%	42.8
20 ng/mL	Biorad Syphilis Level 3	26781	13%	4.2
20 ng/mL	Human Serum	6396	2%	1.0
4 ng/mL	Biorad Chagas	52277	9%	40.1
4 ng/mL	Biorad Syphilis Level 3	6580	2%	5.0
4 ng/mL	Human Serum	1305	1%	1.0
0.8 ng/mL	Biorad Chagas	11149	7%	19.2
0.8 ng/mL	Biorad Syphilis Level 3	1207	15%	2.1
0.8 ng/mL	Human Serum	581	12%	1.0

2.4 Effect of Theranos AP Conjugate Stabilizer

The Theranos AP Conjugate Stabilizer was tested, and the assay performance was comparable to 3% BSA assay buffer.

Table 6: Theranos AP Conjugate Stabilizer

Buffer	Sample	AVG RLU	CV	Modulation
3% BSA	Biorad Chagas	55562	8%	33.9
3% BSA	Seracare Chagas	115546	5%	70.5
3% BSA	Biorad Syphilis Level 3	7482	4%	4.6
3% BSA	Human Serum	1639	5%	1.0
Theranos AP Conjugate Stabilizer	Biorad Chagas	60360	15%	36.3
Theranos AP Conjugate Stabilizer	Seracare Chagas	132185	7%	79.6
Theranos AP Conjugate Stabilizer	Biorad Syphilis Level 3	8952	9%	5.4
Theranos AP Conjugate Stabilizer	Human Serum	1661	24%	1.0

2.5 Effect of Sample Dilutions

The assay sample dilution was tested by comparing 5x, 10x, and 25x sample dilutions. There was acceptable sensitivity at 10x sample dilution.

Table 7: Effect of Sample Dilutions

Sample Dilution	Sample	AVG RLU	CV	Modulation
5X	Biorad Chagas	33415	24%	32.2
5X	Seracare Chagas	69727	3%	67.2
5X	Biorad Syphilis Level 3	5572	18%	5.4
5X	Human Serum	1038	8%	1.0
10X	Biorad Chagas	18791	7%	31.6
10X	Seracare Chagas	40565	2%	68.2
10X	Biorad Syphilis Level 3	3050	5%	5.1
10X	Human Serum	595	12%	1.0
25X	Biorad Chagas	8698	17%	22.3
25X	Seracare Chagas	22874	9%	58.6
25X	Biorad Syphilis Level 3	1289	15%	3.3
25X	Human Serum	390	17%	1.0

2.6 Effect of Incubation Times

The assay incubation time was tested, and the results showed that 10-10-10 gave the best modulations.

Table 8: Effect of Incubation Times

Time	Sample	AVG RLU	CV	Modulation
10_10_10	Biorad Chagas	18791	7%	31.6
10_10_10	Seracare Chagas	40565	2%	68.2
10_10_10	Biorad Syphilis Level 3	3050	5%	5.1
10_10_10	Human Serum	595	12%	1.0
5_5_5	Biorad Chagas	2604	4%	11.5
5_5_5	Seracare Chagas	3596	5%	15.8
5_5_5	Biorad Syphilis Level 3	1200	12%	5.3
5_5_5	Human Serum	227	5%	1.0
2_2_1	Biorad Chagas	638	5%	5.0

2_2_1	Seracare Chagas	545	13%	4.3
2_2_1	Biorad Syphilis Level 3	251	10%	2.0
2_2_1	Human Serum	127	12%	1.0

2.7 Whole Blood and Plasma Screen

There was no interference from whole blood and plasma. The signal from all samples were near background.

Table 9: Whole Blood and Plasma Screen

Sample	AVG RLU	CV
WB 1	525	0%
WB 2	1020	22%
WB 3	427	8%
WB 4	436	18%
WB 5	504	1%
Plasma 1	2484	18%
Plasma 2	2117	9%
Plasma 3	506	36%
Plasma 4	639	7%
Plasma 5	1222	56%

2.8 Effect of HBR

HBR was tested to reduce nonspecific binding from HAMA and RF samples. HBR at 250 ug/mL was able to reduce RF interference.

Table 10: Effect of HBR

			3% BSA		250 ug/mL HBR/3% BSA		
Description	Sample	AVG RLU	CV	Modulation	AVG RLU	CV	Modulation
HAMA	G	6740	10%	6.5	7485	10%	6.6
HAMA	J	6768	17%	6.5	6330	29%	5.6
RF	Rc	13748	8%	13.2	8640	10%	7.6
	Biorad Chagas	33415	24%	32.2	34770	3%	30.7
	Seracare Chagas	69727	3%	67.2	65021	4%	57.4
	Biorad Syphilis Level 3	5572	18%	5.4	5115	4%	4.5
	Human Serum	1038	8%	1.0	1133	4%	1.0

2.9 Cross Reactivity

There was no cross reactivity to RF, HAMA, Anti-Rubella, Anti-CMV, Anti-VZV, Anti-HSV, Anti-EBV, and WNV samples. The diluent used was 3% BSA + 250 ug/mL HBR.

Table 11: Cross Reactivity

Description	Sample	Theranos (Cutoff = 19000)		IBL (positive > 0.65, negative < 0.53)	
RF	R1	2444	negative	0.25	negative
RF	Ra	1926	negative	0.26	negative
RF	Rb	2031	negative	0.18	negative
RF	Rc	13748	negative	0.25	negative
RF	Rd	1847	negative	0.25	negative
HAMA	H	2905	negative	0.27	negative
HAMA	I	1296	negative	0.31	negative
HAMA	L	2882	negative	0.23	negative
HAMA	M	1252	negative	0.27	negative
Control	Biorad Chagas	33415	positive	0.90	positive
Control	Seracare Chagas	69727	positive	1.56	positive
Control	Biorad Syphilis Level 3	5572	negative	0.35	negative
Control	Human Serum	1038	negative	0.48	negative
Cross Reactivity	Anti-Rubella	4784	negative	0.36	negative
Cross Reactivity	Anti-CMV	2951	negative	0.41	negative
Cross Reactivity	Anti-VZV	1424	negative	0.30	negative
Cross Reactivity	Anti-HSV	1662	negative	0.46	negative
Cross Reactivity	Anti-EBV	231	negative	0.06	negative
Cross Reactivity	WNV	1349	negative	0.22	negative

2.10 Blocker Screen

Other blockers (Low Cross, Starting Block) were tested to reduce interference and improve assay performance. Low cross buffer was better than HBR at reducing interference.

Table 12: Effect of Blockers

Blocker	Sample	AVG RLU	CV
No Blocker	1	33263	8%
No Blocker	Biorad Chagas	20446	3%
400 ug/mL HBR	1	29882	21%
400 ug/mL HBR	Biorad Chagas	21655	11%
Low Cross	1	5155	11%
Low Cross	Biorad Chagas	9692	12%
Starting Block	1	24186	11%
Starting Block	Biorad Chagas	13662	7%

2.11 Cutoff Calculation

The cutoff was calculated from testing 6 normal samples using Low Cross Buffer as the diluent. The cutoff was determined to be 13,009 RLU (AVG Normal + 2.5STD).

Table 13: Cutoff Calculation (Low Cross Buffer)

Sample	AVG RLU	CV	S/CO	IBL ELISA
1	11109	7%	0.85	Negative
2	549	11%	0.04	Negative
3	2049	2%	0.16	Negative
4	1854	29%	0.14	Negative
5	1764	6%	0.14	Negative
6	1160	26%	0.09	Negative
Biorad Chagas	16610	7%	1.28	
Seracare Chagas	34891	11%	2.68	
Biorad Syphillis Level 3	2499	19%	0.19	
09/186	114783	14%	8.82	
09/188	126007	16%	9.69	

2.11 Clinical Correlation

There was good clinical correlation between the Theranos system and Abbott Prism. A total of 21 clinical samples were tested.

Table 14: Clinical Correlation

			Theranos	Abbott Prism
Sample	AVG RLU	CV	S/CO	S/CO
1	76727	21%	5.90	2.40
2	39214	12%	3.01	8.40
3	64632	12%	4.97	6.00
4	222977	19%	17.14	6.60
5	48152	27%	3.70	4.80
6	111855	5%	8.60	5.10
7	299022	4%	22.99	10.30
8	65095	5%	5.00	5.60
9	13753	17%	1.06	2.10
10	37191	13%	2.86	3.70
11	66126	12%	5.08	2.60
12	38678	6%	2.97	5.40
13	320	19%	0.02	0.10
14	77748	6%	5.98	9.20
15	108868	8%	8.37	11.10
16	265958	16%	20.44	6.10
17	41041	14%	3.15	5.20
18	55130	8%	4.24	2.20
19	82023	7%	6.31	7.00
20	138448	22%	10.64	9.20
21	148562	21%	11.42	8.20

3. ASSAY SUMMARY

Table 15: Development Summary

Capture Antigen	MyBiosource, MBS537436
Wash Buffer	1X Enzo from 20X
Assay Buffer	Low Cross Buffer
Edison Protocol	Generic2_10X_PSW
Detector Antibody	Novus 2C11 @ 4 ng/mL
Detector Stabilizer	Theranos AP Conjugate Stabilizer
Sample Dilution	10X

4. CLINICAL EVALUATION

More clinical samples need to be tested to further validate the assay. Furthermore, the assay needs to be validated at more than 1 test center. At least 100 or more patients are needed for clinical evaluation.