theranos™
redefining healthcare

Helicobacter pylori Antibody (IgG) Qualitative Assay Development Report

Theranos, Inc

December
2012

Prepared by: Nahal Gharaati

theranos™
redefining healthcare

TABLE OF CONTENTS

[TOC \o "1-3" \h \z \u]**LIST OF TABLES**

[TOC \h \z \c "Table"]

1. ASSAY INFORMATION[TC "ASSAY INFORMATION" \F C \L "2"]

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

This assay is designed to qualitatively determine Helicobacter pylori antibodies (IgG) in human plasma and serum.

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

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

- Inova Diagnostics_QUANTA Lite H. pylori IgG (Cat# 708715)
- BQ Kits_Helicobacter Pylori (H. Pylori) IgG ELISA Kit (Cat# BQ 013G)
  IBL International_Helicobacter pylori IgG ELISA (Cat# IB79236)

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

Helicobacter pylori (IgG) antigen coated surface serves as the capture surface for the H. pylori IgG antibody assay. The sample (plasma or serum) is diluted and then incubated on the capture surface for 10 minutes, the surface is washed, and then an alkaline phosphatase(AP)-labeled anti-human IgG antibody is incubated on the surface for 10 minutes. After the detection antibody incubation, another washing cycle is performed and the alkaline phosphatase substrate is incubated on the surface for 10 minutes, and the resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Antigen	Genway	GWB-T00496
Mouse Anti-Human IgG1 Antibody	Novus Biologicals	NB100-2046
Alkaline Phosphatase Labeling Kit	Dojindo	LK13-10
Theranos In-House Substrate	N/A	N/A
Theranos AP Conjugate Stabilizer	N/A	N/A
Low-Cross Buffer	CANDOR Bioscience	100 500
Blocking Buffer (3% BSA in TBS, 0.05% Sodium Azide)	N/A	N/A

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

1.1 Effect of Capture Antigen Conjugation on Assay Response [TC " Effect of Capture Antigen Conjugation on Assay Response " \f C \l "1"]

A biotin conjugate version and unconjugated versions of the H. pylori antigen were tested on a Microtiter Plate (MTP) as capture surface. The biotin conjugate was coated on an avidin surface followed by blocking. The unconjugated antigen was coated directly followed by a blocking step. The two surfaces were tested against a positive control sample containing H. pylori antibodies and an autoimmune negative control sample obtained from a commercial source along with true normal and H. pylori clinical samples. An anti-human IgG detection antibody AP conjugate was used at a concentration of 50 ng/mL in Blocking Buffer. The response for the directly coated antigen clearly is the better choice to further optimize. The results are summarized in Table 2.

Table [SEQ Table * ARABIC]: Effect of Capture Antigen Surface on assay response.

[LINK Excel.Sheet.12 "\\\\theranos.local\\folders\\Projects\\Experiment Log\\E0700 - E0799\\E0728\\Anti SSA_assay development report.xlsx" "Test Biotin conjugation!R79C2:R88C6" \a \f 4 \h]

	Biotinylated		Mean	St Dev	%CV
negative ctrl	340	184	262	110	42
positive ctrl	8146	8016	8081	92	1
normal #1	912	1234	1073	228	21
clinical #7	24453	24558	24506	74	0
		pos/neg	31		
		clin pos/normal	23		

	Direct Coat		Mean	St Dev	%CV
negative ctrl	624	1096	860	334	39
positive ctrl	114371	114208	114290	115	0
normal #1	3656	3703	3680	33	1
clinical #7	305690	287874	296782	12598	4
		pos/neg	133		
		clin pos/normal	81		

1.2 Capture Antigen Surface Titration [TC " Capture Antigen Surface Antigen " \f C \l "1"]

The H. pylori antigen coated surface was titrated at levels: 5, 2.5 and 1 µg/mL. Table 3 summarizes the results. 5 µg/mL provides good enough modulation between the positive and negative samples as well as lowering the negative background. This was finalized as the capture antigen surface concentration.

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

1 ug/mL	Inter-Cartridge Mean	CV%
Negative (Biorad Ctrl)	183	24
Positive (Biorad Ctrl)	35964	8
Normal (#1)	462	12
Clinical (Strong Pos.) #7	75826	10
Pos control/Neg control	197	
Pos (Clinical)/Neg (Normal)	164	

2.5 ug/mL	Inter-Cartridge Mean	CV%
Negative (Biorad Ctrl)	197	14
Positive (Biorad Ctrl)	161439	24
Normal (#1)	980	19
Clinical (Strong Pos.) #7	349446	10
Pos control/Neg control	818	
Pos (Clinical)/Neg (Normal)	356	

5 ug/mL	Inter-Cartridge Mean	CV%
Negative (Biorad Ctrl)	282	5
Positive (Biorad Ctrl)	299598	24
Normal (#1)	1840	26
Clinical (Strong Pos.) #7	767752	16
Pos control/Neg control	1063	
Pos (Clinical)/Neg (Normal)	417	

10 ug/mL	Inter-Cartridge Mean	CV%
Negative (Biorad Ctrl)	300	1
Positive (Biorad Ctrl)	426290	36
Normal (#1)	2600	3
Clinical (Strong Pos.) #7	1168351	5
Pos control/Neg control	1422	
Pos (Clinical)/Neg (Normal)	449	

1.3 Effect of Detection Conjugate Stabilizer

Two commercial and two in house formulated alkaline phosphatase stabilizers were tested as detection antibody diluents, with the anti-human IgG DAb at 50 ng/mL. Signal modulation was best with the Theranos In-house detection antibody stabilizer. Table 4 summarizes the results.

Table [SEQ Table * ARABIC]: Effect of Detection Conjugate Stabilizer

Stabilzyme	Inter-Cartridge Mean	CV%
Negative Ctrl (Biorad)	442	13
Positive Ctrl (Biorad)	164279	10
Clinical #7 (high)	336815	10
	372	
	763	

In-house Stabilizer	Inter-Cartridge Mean	CV%
Negative Ctrl (Biorad)	258	44
Positive Ctrl (Biorad)	300405	23
Clinical #7 (high)	749649	24
	1163	
	2903	

Biostab	Inter-Cartridge Mean	CV%
Negative Ctrl (Biorad)	662	29
Positive Ctrl (Biorad)	476011	21
Clinical #7 (high)	900301	6
	719	
	1360	

Blocking Buffer	Inter-Cartridge Mean	CV%
Negative Ctrl (Biorad)	274	21
Positive Ctrl (Biorad)	262310	30
Clinical #7 (high)	653263	8
	956	
	2382	

1.4 Detection Conjugate Titration

The AP conjugated detection antibody was titrated in the Theranos detection conjugate stabilizer. The best modulation between the positive and negative control was achieved with 50 ng/mL of the anti-IgG Dab. This concentration yielded enough modulation while still keeping the background very low and therefore was chosen for the rest of this assay's development.

Table [SEQ Table * ARABIC]: Detection Conjugate Titration

25 ng/mL	Inter-Cartridge Mean	CV%
Negative Ctrl (Biorad)	442	
Positive Ctrl (Biorad)	164279	
Clinical #7 (high)	336815	
	372	
	763	

50 ng/mL	Inter-Cartridge Mean	CV%
Negative Ctrl (Biorad)	417	19
Positive Ctrl (Biorad)	322277	10
Clinical #7 (high)	652951	3
	773	
	1566	

100 ng/mL	Inter-Cartridge Mean	CV%
Negative Ctrl (Biorad)	582	5
Positive Ctrl (Biorad)	510161	10
Clinical #7 (high)	1196614	9
	876	
	2056	

200 ng/mL	Inter-Cartridge Mean	CV%
Negative Ctrl (Biorad)	1578	14
Positive Ctrl (Biorad)	879942	12
Clinical #7 (high)	1533255	6
	558	
	971	

1.5 Effect of Diluent Buffer

The effect of diluent buffer was tested with four different blocking buffers. The controls used were a known high RLU normal sample and a known high clinical sample. The results showed that the Low Cross Buffer was the best at lowering the normal sample in this assay in response to antibodies to H. pylori IgG. At the same time, this buffer maintained the high response for the positive clinical sample. Therefore, Low Cross Buffer was chosen as the diluent for this assay. Table 6 summarizes the data.

Table 6: Effect of Diluent Buffer

Blocking Buffer	Inter-Cartridge Mean	CV%
Normal #20 (negative)	6095	16
Clinical #7 (high pos. sample	654456	17
	107	

Surmodics	Inter-Cartridge Mean	CV%
Normal #20 (negative)	3977	16
Clinical #7 (high pos. sample	486100	23
	122	

Low Cross	Inter-Cartridge Mean	CV%
Normal #20 (negative)	2845	5
Clinical #7 (high pos. sample	554403	12
	195	

Super Block	Inter-Cartridge Mean	CV%
Normal #20 (negative)	4182	6
Clinical #7 (high pos. sample	572224	19
	137	

1.6 Effect of Sample Dilution[TC "Effect of Sample dilution" \f C \l "1"]

The effect of sample dilution was tested with final sample dilution factors of 1:10, 1:25 and 1:50 and 1:100 into 3% BSA in TBS blocking buffer. Modulation between pooled positive and negative sera was best at 50 fold sample dilution, as a result of a greater reduction in the signal from negative samples compared to the reduction in signal from the positive samples. Therefore, the 50X Sample Dilution Protocol is the one we will continue with for this assay. Results are summarized in Table 7.

Table 7: Effect of Sample Dilution

10X	Inter-Cartridge Mean	CV%
Normal #20 (negative)	2845	5
Clinical #7 (high pos. sample)	554403	12
	195	

25X	Inter-Cartridge Mean	CV%
Normal #20 (negative)	1243	16
Clinical #7 (high pos. sample)	329966	17
	265	

50X	Inter-Cartridge Mean	CV%
Normal #20 (negative)	707	18
Clinical #7 (high pos. sample)	239170	5
	338	

100X	Inter-Cartridge Mean	CV%
Normal #20 (negative)	427	14
Clinical #7 (high pos. sample)	126369	16
	296	

1.7 Effect of Changing Reagent Incubation Time and PSW [TC "Effect of changing reagent incubation time" \f C \l "1"]

The effect of shorter reagent incubation times was tested with the sample, detection conjugate and substrate incubation times respectively of 10, 10, 10; 5, 5, 5; and 2, 2, 1 minutes. At the same time, the Post Sample Wash (PSW) step was excluded to see if any affect is seen in the overall assay performance. Although there was a slight drop in background which increased the assay modulation, it was decided to keep the PSW step in the protocol to keep consistent with other assays and give flexibility of future multiplexing. The best response for sample incubation times was at chosen at this time to remain at the 10, 10, 10 minute incubation protocol, however, if needed it is possible to use the 5, 5, 5 protocol to once again possibly combine assays for multiplexing.

.

Table 8: Effect of Changing Reagent Incubation Time and PSW

10, 10, 10	Inter-Cartridge Mean	CV%
Normal #20 (negative)	707	18
Clinical #7 (high pos. sample)	239170	5
pos/neg	338	

10, 10, 10 (no PSW)	Inter-Cartridge Mean	CV%
Normal #20 (negative)	578	9
Clinical #7 (high pos. sample)	198174	12
pos/neg	343	

5, 5, 5	Inter-Cartridge Mean	CV%
Normal #20 (negative)	254	10
Clinical #7 (high pos. sample)	46324	8
pos/neg	183	

2, 2, 1	Inter-Cartridge Mean	CV%
Normal #20 (negative)	102	8
Clinical #7 (high pos. sample)	4110	17
pos/neg	40	

1.8 Normal Sample Screen: Cut-off Determination

Normal donor plasma (N=20) were obtained and tested on the three commercial ELISA kits and on the Theranos System. The Theranos cut-off value was determined by taking the mean RLU of the normal samples plus 10 times the standard deviation of the 20 normal samples (Table 10). The sample RLU divided by the cut-off value yields the Antibody Index. The following criteria was applied to categorize the result as positive (red), negative (green) or borderline (yellow).

Ab index	Sample RLU/Cut off
Ab index > 1.1	Positive
Ab index > 0.9 < 1.1	Equivocal/Borderline
Ab index < 0.9	Negative

Table 9: Normal Sample Screen: Theranos vs. 3 Commercial H. pylori_IgG ELISAs

Samples	Inter-Cartridge Mean	Inter-Cartridge CV%	Theranos Ab Index 10*STDEV	INOVA kit Units	BQ kit Units	IBL International U/mL
Normals (#1)	652	23	0.09	2	0.07	0.12
Normals (#2)	547	10	0.08	3	0.09	0.23
Normals (#3)	824	12	0.11	5	0.12	0.24
Normals (#4)	1066	29	0.15	3	0.12	0.19
Normals (#5)	861	11	0.12	9	0.16	0.33
Normals (#6)	687	18	0.09	8	0.16	0.34
Normals (#7)	606	37	0.08	3	0.08	0.25
Normals (#8)	3300	6	0.45	10	0.23	0.32
Normals (#9)	616	28	0.08	3	0.08	0.15
Normals (#10)	693	24	0.10	8	0.25	0.33
Normals (#11)	978	15	0.13	6	0.16	0.27
Normals (#12)	682	22	0.09	2	0.39	0.16
Normals (#13)	606	21	0.08	7	0.08	0.18
Normals (#14)	825	20	0.11	3	0.16	0.48
Normals (#15)	1954	22	0.27	2	0.16	0.31
Normals (#16)	876	16	0.12	2	0.41	0.13
Normals (#17)	1085	3	0.15	8	0.22	0.20
Normals (#18)	895	20	0.12	3	0.41	0.39
Normals (#19)	665	8	0.09	8	0.08	0.21
Normals (#20)	1156	13	0.16	1	0.21	0.41
MEAN	979					
CUT OFF	7272	10*STDEV				

1.9 Clinical Sample Correlation

N=34 samples obtained from patients with gastritis ulcer were tested on the Theranos H. pylori_IgG assay. The same samples were run on three commercial H. pylori ELISAs and the correlation of the results to the Theranos assay is reported in Table 10 below. Good correlation was seen for most samples. In general, the BQ commercial kit did not track as well as the other predicate methods used.

Table 10: Clinical Sample Screen: Theranos vs. 3 Commercial H. pylori_IgG ELISAs

						10*STDEV
	Mean RLU	CV (%)	INOVA	BQ	IBL	Theranos
Sample			Units	Units	U/mL	Ab Index
CI01	16629	8	32.5	0.97	1.59	2.3
CI02	106785	17	128.4	3.02	2.39	14.7
CI03	121471	8	43.9	0.91	1.98	16.7
CI04	106361	15	44.0	0.91	1.86	14.6
CI05	58291	28	61.9	1.94	2.15	8.0
CI06	109997	9	30.5	1.08	1.95	15.1
CI07	239170	5	143.3	3.09	2.58	32.9
CI08	8952	5	43.2	1.26	1.33	1.2
CI09	273998	8	80.8	2.18	2.49	37.7
CI10	65144	9	16.8	0.73	1.29	9.0
CI11	31908	8	33.4	1.19	1.64	4.4
CI12	347859	14	144.4	3.13	2.62	47.8
CI13	90265	23	40.7	0.84	1.93	12.4
CI14	398081	18	145.2	3.11	2.64	54.7
CI15	163405	5	130.8	3.07	2.50	22.5
CI16	177040	20	38.1	0.73	2.19	24.3
CI17	149028	2	45.6	0.88	2.00	20.5
CI18	451786	14	145.6	3.23	2.64	62.1
CI19	35238	42	122.8	2.88	2.11	4.8
CI20	62668	22	121.7	2.91	2.25	8.6
CI21	28213	12	83.2	1.96	1.57	3.9
CI22	97115	23	112.1	2.61	2.13	13.4
CI23	129145	3	69.9	2.33	2.40	17.8
CI24	112093	3	42.6	0.82	1.93	15.4
CI25	364396	7	140.9	3.12	2.66	50.1
CI26	82713	15	42.7	0.76	1.85	11.4
CI27	34283	26	64.5	2.05	1.89	4.7
CI28	148340	22	99.3	2.41	2.23	20.4
CI29	84895	12	88.4	2.43	2.29	11.7
CI30	7816	15	27.7	0.77	1.00	1.1
CI31	93622	14	88.5	1.87	2.07	12.9
CI32	107219	17	55.3	2.00	2.41	14.7
CI33	136232	11	41.6	0.69	2.00	18.7
CI34	245687	15	65.9	2.13	2.50	33.8

1.10 Specificity

The specificity of the Theranos H. pylori_IgG assay was tested against a few known bacterial species mentioned in literature to possibly cross-react with Helicobacter pylori. Positive control samples for Campylobacter jejuni and Borrelia burgdorferi were obtained and tested on the Theranos system.

Theranos H. pylori IgG assay does not demonstrate cross reactivity against the two bacteria tested below in Table 11.

Table [SEQ Table * ARABIC]: Specificity

X-Reactivity Test	Inter-Cartridge Mean	CV%
Normal #20 (negative)	1124	8
Clinical #9 (high pos. sample)	243986	33
Campylobacter jejuni	416	20
Borrelia burgdorferi	416	25
pos/neg	217	

1.11 Rf and HAMA Positive Sample Testing

10 Rf positive and 10 HAMA positive sera obtained from a commercial source were tested on the Theranos H. pylori_IgG assay. The same samples were also tested in the CLIA lab. Out of the total 20 samples tested, all were in agreement in each test but 1 Rf sample that disagreed and 1 HAMA sample that disagreed. There were also 2 HAMA samples that gave equivocal results in either test.

Table [SEQ Table * ARABIC]: Rf and HAMA positive sample screen

Samples	Inter-Cartridge Mean	CV%	CLIA Results (U/mL)	Theranos Ab Index 10*STDEV
Rf - 11	17717	17	1.28	2.44
Rf - 12	3288	10	0.495	0.45
Rf - 13	658	14	0.4	0.09
Rf - 14	3712	7	1.42	0.51
Rf - 15	351	25	0.4	0.05
Rf - 16	647	16	0.468	0.09
Rf - 17	11851	5	1.24	1.63
Rf - 18	5929	11	0.688	0.82
Rf - 19	126700	26	>8.00	17.42
Rf - 20	513	14	0.488	0.07

Samples	Inter-Cartridge Mean	CV%	CLIA Results (U/mL)	Theranos Ab Index 10*STDEV
Hama - 11	730	24	0.471	0.10
Hama - 12	1623	30	0.859	0.22
Hama - 13	1827	13	0.936	0.25
Hama - 14	217551	21	4.51	29.92
Hama - 15	7232	23	1.49	0.99
Hama - 16	11076	12	0.764	1.52
Hama - 17	54778	16	>8.00	7.53
Hama - 18	168503	11	>8.00	23.17
Hama - 19	56366	19	6.41	7.75
Hama - 20	129340	20	4.08	17.79

1.12 Whole Blood Screening

The H. pylori_IgG assay was tested with 5 matched Male and 5 matched Female samples to see effects of Whole Blood versus Serum and Plasma (Lithium Heparin and EDTA) samples. Only one Male patient in this experiment would result in a positive patient if serum/plasma was used to test. However, for this same person, whole blood test showed a negative result.

Table [SEQ Table * ARABIC]: Whole Blood, Serum and Plasma Results

[SHAPE * MERGEFORMAT]

1.13 Different Matix Effects

The H. pylori_IgG assay was then tested against 6 samples of grossly hemolyzed, icteric and lipemic samples to see possible effects of these various matrices on this assay. Out of 18 total samples tested, only 1 hemolyzed and 1 icteric sample showed a negative result on Theranos assay system but was given a low positive signal from the CLIA lab results.

Table [SEQ Table * ARABIC]: Hemolyzed vs. Icteric vs. Lipemic Matrices

Hemolyzed			
Sample	Theranos	CLIA	
H1	7.68	>8.00	U/mL
H2	0.77	1.30	U/mL
H3	0.10	0.57	U/mL
H4	0.06	0.42	U/mL
H5	0.23	0.96	U/mL
H6	0.15	0.62	U/mL

Icteric			
Sample	Theranos	CLIA	
H7	25.44	6.61	U/mL
H8	0.47	0.76	U/mL
H9	4.18	2.17	U/mL
H10	12.74	>8.00	U/mL
H11	0.21	1.39	U/mL
H12	1.08	1.23	U/mL

Lipemic			
Sample	Theranos	CLIA	
H13	0.29	0.43	U/mL
H14	0.14	< 0.40	U/mL
H15	20.05	3.51	U/mL
H16	0.49	0.40	U/mL
H17	21.33	4.43	U/mL
H18	0.13	< 0.40	U/mL