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Hepatitis C Virus IgG Assay Feasibility Report

Theranos, Inc.

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

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

This assay is designed to qualitatively determine the presence of IgG antibodies to Hepatitis C Virus (HCV) in human 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:

- US Biological, Cat # H1920-17J
- Abnova, Cat # KA0291

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

A recombinant protein containing the HCV core, NS3, NS4 and NS5 immunodominant regions serves as the capture surface for Hepatitis C Virus IgG antibodies in the sample. After incubation of the appropriately-diluted sample on the capture surface, the surface is washed. A mouse anti-human IgG detection antibody is incubated on the surface. After this incubation period, the surface is washed again. Alkaline phosphatase substrate is incubated on the surface, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Carbonate-bicarbonate buffer	Sigma	C3041
Heterophilic Blocking Reagent (HBR)	Scantibodies	3KC533
Mouse Anti-Human IgG Antibody	Novus	NB100-2046
USBiological HCV IgG Kit	USBiological	H1920-17J
Axell(Accurate Chemical and Scientific Corp)- HCV IgG Kit	Axell	BMDEU1039
Abnova HCV IgG Kit	Abnova	KA0291
Biochain HCV IgG Kit	Biochain	Z7010002
Phospho Glo Substrate	KPL	55-60-04

Table [SEQ Table * ARABIC]: Antigen List

Antigen #	Vendor	Cat#	Description
1	Mybiosource	MBS319415	HCV Core, NS3, NS4, NS5 recombinant protein
2	Mybiosource	MBS319416	HCV Core, NS3, NS4, NS5 recombinant protein
3	Mybiosource	MBS319234	Core antigen (1-120)
4	Mybiosource	MBS538362	HCV antigen, Rh, core
5	USBiological	H1919-85	HCV nucleocapsid, NS3 genotype 1b, NS4 genotype 1b and 1a, and NS5 genotype 1b and 1a
6	USBiological	H1919-85E	Hepatitis C Virus, nucleocapsid, NS3, NS4, and NS5 recombinant

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

2.1 Capture Surface: Antigen Screen on MTP

Commercially available antigens were screened with positive and negative controls from the Abnova Kit in addition to normal sera from Bioreclamation. Antigen was directly coated on Nunc 384 microtiter (MTP) plates at a concentration of 10 ug/mL in carbonate-bicarbonate buffer. A sample dilution of 1:25 was added to the surface followed by wash steps. Antigen # 2 was chosen based on best modulation between the positive control and the mean of normals. Normal Sera was obtained from Bioreclamation. The detection antibody concentration was set at 100ng/ml in regular 3% BSA blocking buffer.

Table [SEQ Table * ARABIC]: Capture Surface Screen on MTP

Sample Type	Sample #	Antigen #1 RLU	Antigen #2 RLU	Antigen #3 RLU	Antigen #4 RLU
Pos CTL	Abnova Kit	1009	3950	4040	1150
Neg CTL	Abnova Kit	419	432	1369	690
Modulation +/-		2.4	9.1	3.0	1.7
Negative Samples (Normal Sera)	BRH351243	489	554	820	915
	BRH351244	427	436	755	721
	BRH351245	382	344	1250	522
	BRH351264	415	682	929	530
	BRH351265	365	310	1122	755
	BRH351267	427	419	1086	724
	BRH351268	328	303	1126	472
	BRH351269	475	585	1338	678
	BRH351272	384	393	1155	552
	BRH351281	351	356	668	568
	BRH351282	419	430	1314	592
	BRH351283	314	385	672	483
	BRH351291	438	417	1345	437
Neg Mean RLU		401	432	1045	611
Modulation Pos CTL/Mean Neg Samples		**2.5**	**9.1**	**3.9**	**1.9**

2.2 Capture Surface Titration on the Theranos system

The capture surface Antigen #2 was titrated at the following concentrations: 20ug/ml, 10ug/ml, 5ug/ml, 2.5ug/ml and 1.25ug/ml. Both positive and negative controls from the Abnova kit were used for this screening. The blank refers to the background control when no sample is added. The protocol does a final sample dilution of 1:25 while detection antibody is maintained at 100ng/ml in regular 3% BSA blocking buffer.

The optimal antigen concentration which was determined to be 2.5ug/ml was then further tested with normal clinical sera to determine modulation. Normal Sera was obtained from Bioreclamation.

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

[Antigen2] ug/ml	Sample Type	Mean RLU	CV%	Modulation
20	Pos CTL	75436	9.8	4.5
	Neg CTL	16860	15.4	
	Blank	3628	23.4	
10	Pos CTL	43540	4.9	4.6
	Neg CTL	9489	11.4	
	Blank	3103	17.9	
5	Pos CTL	18790	4.2	6.7
	Neg CTL	2791	21.4	
	Blank	2075	5.8	
2.5	Pos CTL	13741	18.4	11.5
	Neg CTL	1190	9.8	
	Blank	743	12.7	
1.25	Pos CTL	4573	24.9	11.2
	Neg CTL	407	14.2	
	Blank	320	17.1	
0.5	Pos CTL	2258	25.4	6.9
	Neg CTL	328	10.6	
	Blank	247	25.0	

Table [SEQ Table * ARABIC]: Testing Antigen 2 at 2.5ug/ml with Normal Clinical Sera-Theranos system

Sample Type	Sample #	Mean RLU	CV%
Pos CTL	Abnova Kit	13741	18.4
Neg CTL	Abnova Kit	1190	9.8
Modulation +/-		11.5	
Negative Samples (Normal Sera)	BRH351243	1389	25.5
	BRH351244	1849	7.0
	BRH351245	1239	18.9
	BRH351264	4844	9.5
	BRH351265	2759	19.7
	BRH351267	2379	7.0
	BRH351268	1489	21.3
	BRH351269	3199	16.2
	BRH351272	1646	6.6
	BRH351281	2065	13.7
Neg Mean RLU		2286	
Modulation :Pos CTL/ Mean Neg Samples		6.0	

2.3 Detection Antibody Screen

Two different types of detection antibodies were evaluated for optimal modulation. The mouse-anti human was found to be ideal for this assay and will be used from here onwards. The Goat-Anti Human detection antibody was tested at a 1:10K dilution from stock (as per manufacturer's instruction) and the Mouse-Anti Human was tested at 100ng/ml in regular 3% BSA blocking buffer.

Table [SEQ Table * ARABIC]: Detection Antibody Screen

Detection Antibody	Sample Type	Mean RLU	CV%	Modulation
Goat- Anti Human IgG	Pos CTL	299754	15.3	7.1
	Neg CTL	42068	10.8	
	Blank	41075	21.6	
Mouse-Anti Human IgG	Pos CTL	13741	18.4	11.5
	Neg CTL	1190	9.8	
	Blank	743	12.7	

2.4 Specificity on MTP

While antigen#2 was the optimal antigen in terms of modulation, cross reactivity can be a significant issue. As new antigens and positive controls were obtained in house, MTP screening was resumed. Antigens 5 and 6 were tested along with Antigen 2 for both modulation and cross reactivity. The antigen concentration was maintained at 2.5ug/ml and detection antibody at 100ng/ml in 3% BSA blocking buffer. Cross reactivity with Rubella IgG (RV) positive clinical samples was tested. Overall in terms of modulation and cross reactivity studies, antigen 2 at 2.5ug/ml is still the best antigen to move forward. Normal Sera was obtained from Bioreclamation.

Table [SEQ Table * ARABIC]: Cross Reactivity-MTP Set 1

Sample Type	Sample #		Antigen #2 RLU	Antigen #5 RLU	Antigen #6 RLU
Pos CTL	Abnova Kit		38698	18566	25732
Pos CTL	US Biological Kit		89139	52599	73948
Pos Mean RLU			**63918**	**35583**	**49840**
Negative Samples (Normal Sera)	BRH351249		2271	6220	5684
	BRH351250		1502	2535	1646
	BRH351251		1045	2180	1384
	BRH351252		1400	4122	3395
	BRH351272		1821	3099	5855
	BRH351291		1135	1857	378
	BRH351244		1014	2987	2001
	BRH351268		772	1901	1589
	BRH351264		5590	1956	1383
Neg Mean RLU			**1839**	**2984**	**2591**
Modulation Mean Pos CTL/Mean Neg Samples			**35**	**12**	**19**
Level	(IU/ml)				
RV IgG Pos Clinical Samples :	16	W070510215376	1655	3831	2047
	21	W070510215367	1185	3819	2413
	13	W070510215359	804	7101	9849
	122	W070510215369	915	3539	2037
Mean RV IgG Pos Clinicals			**1140**	**4572**	**4086**

2.5 Alkaline Phosphatase Stabilizer

Two commercial alkaline phosphatase stabilizers and the In-House AP stabilizer were tested as detection antibody (DAb) diluents. The In House AP stabilizer is prepared by adding 0.1mM zinc chloride and 5mM magnesium chloride to the 3% BSA blocking buffer. The IgG DAb concentration was tested at 100ng/ml in In-House AP Stabilizer and Stabilizyme AP while 25ng/ml of DAb IgG was tested in Biostab. Antigen concentration was maintained at 2.5ug/ml and the sample gets diluted 25 fold after protocol run. The positive controls were obtained from the USBiological and Abnova Kit while the negative control was sera from a normal clinical sample (BRH351250).

Table [SEQ Table * ARABIC]: Alkaline Phosphatase Stabilizers

Detection Stabilizer	Sample Type	Mean RLU	CV%	Modulation
In House AP Stabilizer	US Biol Pos CTL	85582	10.0	47
	Abnova Pos CTL	19873	13.9	11
	Neg CTL	1821	7.2	
Stabilizyme AP	US Biol Pos CTL	58088	10.0	16
	Abnova Pos CTL	21433	3.9	6
	Neg CTL	3604	14.8	
Biostab	US Biol Pos CTL	65408	20.1	25
	Abnova Pos CTL	16620	27.7	6
	Neg CTL	2566	51.8	

2.6 Detection Antibody Titration

The AP conjugated detection antibodies were titrated in In House AP Stabilizer. The best modulation between the positive and negative controls was observed at 100ng/ml final. The positive controls were obtained from the US Biological and Abnova Kit while the negative control is sera from a normal clinical sample.

Table [SEQ Table * ARABIC]: Detection Antibody Titration

[DAb IgG], ng/ml	Sample Type	Mean RLU	CV%	Modulation
25	US Biol Pos CTL	19624	18.3	22
	Abnova Pos CTL	4940	13.0	5
	Neg CTL	906	22.9	
50	US Biol Pos CTL	43932	8.1	28
	Abnova Pos CTL	9668	7.8	6
	Neg CTL	1575	17.1	
100	US Biol Pos CTL	85582	10.0	47
	Abnova Pos CTL	19873	13.9	11
	Neg CTL	1821	7.2	
150	US Biol Pos CTL	132587	5.1	35
	Abnova Pos CTL	31329	10.9	8
	Neg CTL	3839	8.8	

2.7 Incubation Times

The effect of shorter reagent incubation times was tested with sample, detection conjugate and substrate incubation times respectively of 10, 10, 10 and 5, 5, 5 minutes. Assay modulation was excellent at the 10,10,10 minute incubation times while modulation fell off sharply at the 5,5,5 incubation time. The positive controls were obtained from the USBiological and Axell kits while the negative control was from the Abnova Kit. Here, antigen concentration is 2.5ug/ml while detection antibody is 100ng/ml in In-house AP Stabilizer.

Table [SEQ Table * ARABIC]: Incubation Time

Incubation Times	Sample Type	Mean RLU	CV%	Modulation
5-5-5 Protocol	US Biological Pos CTL	15271	24.1	29
	Axell Pos CTL	14871	13.8	28
	Abnova Neg CTL	523	19.5	
10-10-10 Protocol	US Biological Pos CTL	84109	9.5	161
	Axell Pos CTL	84474	18.3	161
	Abnova Neg CTL	523	20	

2.8 Sample Dilution

The effect of sample dilution was tested with final sample dilution factors of 1:25 and 1:50 into 3% BSA blocking buffer. Modulation between positive controls and negative control sera was best at 1:25. The positive controls were obtained from USBiological, Axell and Abnova kits. Normal Sera was obtained from Bioreclamation. Antigen concentration is set at 2.5ug/ml while detection antibody is 100ng/ml in In-house AP Stabilizer.

Table [SEQ Table * ARABIC]: Sample Dilution

Sample Dilution		**1:50**	**1:25**
Sample Type	**Sample #**	**MEAN RLU**	**MEAN RLU**
Pos CTL	US Biological Kit	34410	84109
Pos CTL	Axell Kit	23789	84474
Low Pos CTL	Abnova Kit	9198	22886
Pos Mean RLU		22466	63823
Negative Samples(Normal Sera)	BRH351242	618	1100
	BRH351247	968	1676
	BRH351248	564	981
	BRH351249	1054	1432
	BRH351250	773	1741
	BRH351251	496	886
	BRH351252	1326	2560
	BRH351253	1985	5033
	BRH351254	5249	10497
	BRH351255	2126	3276
	BRH351256	1082	1735
	BRH351258	886	2835
	BRH351263	164	909
Neg Mean RLU		1330	2666
Mean Pos / Mean Neg		16.9	23.9

2.9 Effect of the Heterophilic Blocking Reagent (HBR)

The addition of HBR in the 3% BSA blocking buffer sample diluent was tested in this assay. HBR is typically used to help eliminate false positives. No significant improvements were observed upon addition of HBR at the recommended concentration of 400ug/ml. Moreover, the addition of HBR dropped the modulation from 29.7 to 22.7 and there was no impact on the mean negative RLU as well. Antigen concentration is set at 2.5ug/ml while detection antibody is at 100ng/ml in In-house AP Stabilizer.

Table [SEQ Table * ARABIC]: Effect of HBR on Normal Clinical Sera

[HBR], ug/mL:		0	400
Sample Type	Sample #	Mean RLU	Mean RLU
Pos CTL	USBiological Kit	84109	64641
Pos CTL	Biochain Kit	79035	70852
Pos CTL	Axell Kit	84474	57898
Low Pos CTL	Abnova Kit	22886	15247
Pos Mean RLU		**67626**	**52159**
Negative Samples(Normal Sera)	BRH351242	1100	1063
	BRH351247	1676	1751
	BRH351248	981	831
	BRH351249	1432	2109
	BRH351250	1741	1155
	BRH351251	886	706
	BRH351252	2560	3288
	BRH351253	5033	4037
	BRH351254	10497	9491
	BRH351255	3276	4016
	BRH351256	1735	2270
	BRH351258	2835	1238
	BRH351263	909	2885
	BRH351266	851	926
	BRH351270	1760	1100
	BRH351271	947	2188
	BRH351274	1399	909
	BRH351275	1376	1408
Neg Mean RLU		**2277**	**2298**
Mean Pos RLU/Mean Neg RLU		**29.7**	**22.7**

2.10 Specificity tests on the Theranos System-HAMA and Rf samples

Positive disease samples known to cause false positives in this HCV IgG assay were tested on the Theranos system. Both rheumatoid factor (Rf) and Human Anti-Mouse Antibodies (HAMA) positive samples were tested with and without HBR on the Theranos system. The HBR did not have any significant positive effect in this assay and was not used further. One sample out of the 6 Rf positive clinical samples tested, consistently gave high levels of cross reactivity on the Theranos system. This sample was also positive in both the commercial kits indicating that this sample could indeed be positive for HCV IgG.

A general cutoff value for the Theranos system was determined by taking the mean RLU of the normal samples plus 5 times the standard deviation of the normal samples. Based on this equation, HAMA Sample #4 came out borderline positive on the Theranos system. This sample also came out positive in the Abnova Kit. Only one out of the sixteen HAMA samples ran on the Theranos system resulted in a borderline positive result. Here, antigen concentration is 2.5ug/ml while detection antibody is 100ng/ml in In-house AP Stabilizer.

Table [SEQ Table * ARABIC]: Specificity Test on the Theranos System

[HBR], ug/mL:		**0**	**400**
Sample Type	Sample #	Mean RLU	Mean RLU
Pos CTL	USBiological Kit	84109	64641
Pos CTL	Biochain Kit	79035	70852
Pos CTL	Axell Kit	84474	57898
Low Pos CTL	Abnova Kit	22886	15247
Pos Mean RLU		**67626**	**52159**
Neg Mean RLU*		**2277**	**2298**
Mean Pos RLU/Mean Neg RLU		**29.7**	**22.7**
HAMA Positive Clinicals	2	12286	12879
	3	568	630
	4	22148	22289
	6	11548	13932
	8	7151	7372
	9	6884	8866
RF Positive Clinicals	1	3374	2852
	2	960	815
	3	1358	1391
	4	830	854
	5	5439	5178
	6	867094	523371

*Neg Mean RLU –Refers to the Normal Sera Mean RLU reported based on Table 12.

Table [SEQ Table * ARABIC]: Kit comparison data

Sample Type	US Biological Kit	Abnova Kit	Theranos System*
US Biol Pos CTL	+	+	+
Biochain Pos CTL	+	+	+
Axell Pos CTL	+	+	+
Abnova Pos CTL	+	+	+
Neg CTL	-	-	-
BRH351242	-	-	-
BRH351247	-	-	-
BRH351248	-	-	-
BRH351249	-	-	-
BRH351250	-	-	-
BRH351251	-	-	-
BRH351252	-	-	-
BRH351253	-	-	-
BRH351254	+	+	-
BRH351255	-	-	-
BRH351256	-	-	-
BRH351258	-	-	-
BRH351263	-	-	-
BRH351266	-	-	-
HAMA #2	-	+	-
HAMA #4	-	+	+
HAMA #6	+	+	-
RF #6	+	+	+

*** Based on a generic cut off equation:**

Cutoff value = Mean (RLU normal samples) + (5 x StDev (RLU Normal Samples))

Table [SEQ Table * ARABIC]: Sixteen HAMA Samples Screen

Sample Type	Sample #	Mean RLU
Pos CTL	USBiological Kit	84109
Pos CTL	Biochain Kit	79035
Pos CTL	Axell Kit	84474
Low Pos CTL	Abnova Kit	22886
Pos Mean RLU		**67626**
Neg Mean RLU		**2277**
Mean Pos RLU/Mean Neg RLU		**29.7**
HAMA Positive Clinical Samples	#2	12286
	#3	568
	#4	22148
	#6	11548
	#8	7151
	#9	6884
	#11	5547
	#13	12825
	#14	9202
	#15	1550
	#17	5008
	#20	9253
	#21	2209
	#22	1838
	#23	1351
	#24	4376

2.11 Specificity tests on the Theranos System-WHO Controls

WHO controls representing conditions unrelated to HCV infection and containing potentially interfering substances were tested on the Theranos system. The antigen concentration is set at 2.5ug/ml while detection antibody is 100ng/ml in In House AP stabilizer. WHO antibody controls for anti-HBV, anti-HAV, anti-HIV-1/2, anti HSV and anti-CMV were all found to not cross react on the Theranos system. Additionally, positive antibody kit controls for diseases such as *Treponema pallidum and Influenza A* were also found to not cross react in this assay. The WHO quality control standard for HCV was obtained and evaluated on the Theranos system. While the control is not used to evaluate sensitivity of the assay, the result obtained was ~10 fold higher than the mean negative control sera.

Sample Type	**Mean RLU**	**CV%**
Biochain Pos CTL	54366	8.2
Abnova Pos CTL	20957	14.6
Mean Pos RLU	**37662**	
Abnova Neg CTL	335	11.6
BRH351242	1076	15.9
BRH351247	1640	8.7
BRH351249	1369	15.5
BRH351252	3357	11.8
BRH351255	3469	13.9
BRH351256	2358	11.8
BRH351258	1158	14.9
BRH351263	1780	13.9
BRH351266	551	13.4
BRH351270	951	6.8
Mean Negative RLU	**1771**	
Mean Pos RLU/Mean Neg RLU	**21**	
Hepatitis B WHO Control	1671	10.2
Hepatitis A WHO Control	3137	16.2
Human immunodeficiency virus 1/2 WHO Control	3145	9.5
Cytomegalovirus WHO Control	2513	4.2
Herpes simplex virus WHO Control	1173	14.6
Treponema pallidum- Kit Pos CTL	1661	19.3
Influenza A- Kit Pos CTL	424	19.9

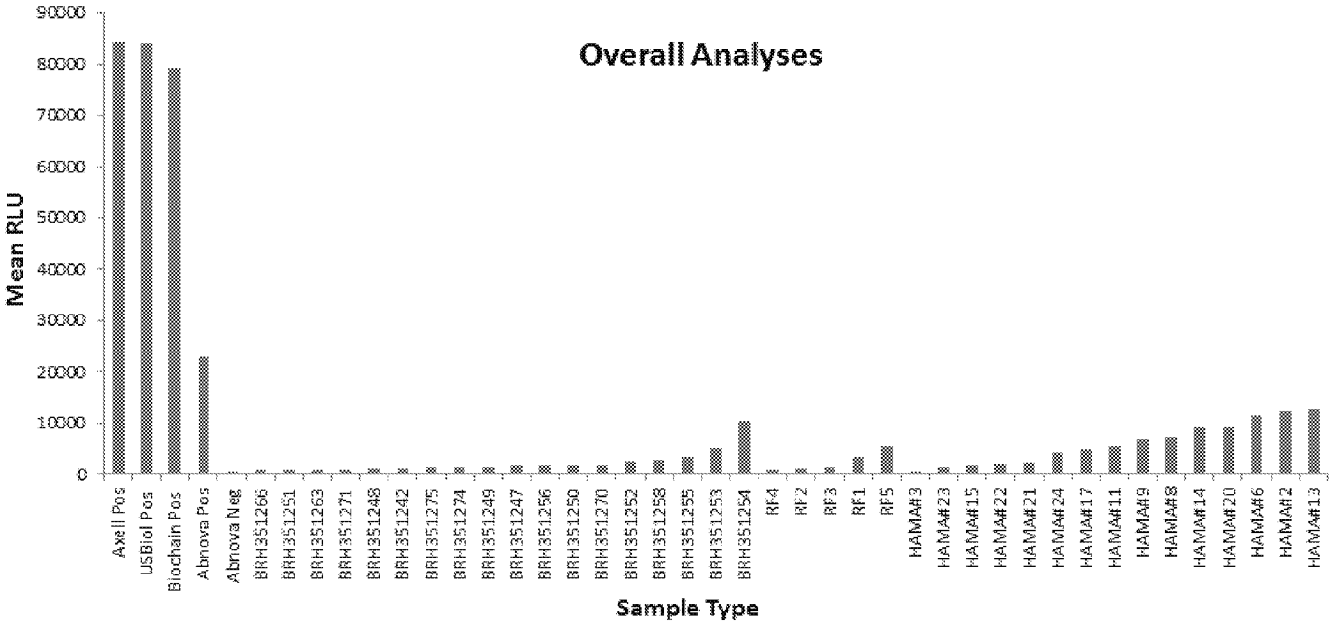
Table [SEQ Table * ARABIC]: Evaluating the HCV WHO Control

Sample Type	**Mean RLU**	**CV%**
Biochain Pos CTL	54366	8.2
Abnova Pos CTL	20957	14.6
Mean Pos RLU	**37662**	
Hepatitis C WHO Control Pos CTL	13727	3.8
Abnova Neg CTL	335	11.6
BRH351242	1076	15.9
BRH351247	1640	8.7
BRH351249	1369	15.5
BRH351250	1189	18.9
BRH351251	770	18.7
BRH351252	3357	11.8
BRH351255	3469	13.9
BRH351256	2358	11.8
BRH351258	1158	14.9
BRH351263	1780	13.9
BRH351266	551	13.4
BRH351270	951	6.8
BRH351271	1425	19.4
BRH351274	848	22.9
BRH351275	1080	18.9
Mean Negative RLU	1535	
WHO HCV CTL Pos CTL/Mean Neg RLU	9	

2.12 Summary of the HCV assay on the Theranos system

A graphical representation demonstrating the HCV assay's performance on the Theranos system. HAMA Sample #4 is not included in the figure. Here, antigen concentration is 2.5ug/ml while detection antibody is 100ng/ml in In-house AP Stabilizer.

Figure [SEQ Figure * ARABIC]: Overall analysis of Antigen 2



2.13 Stability Studies

Stability monitoring is ongoing for the the assay reagents stored at 4°C and protected from light. Different detection antibody stabilizers will be evaluated throughout this study. These include detection antibody in either Stabilzyme AP or the Theranos In-house AP stabilizer. Stabilzyme AP works best compared to the In House AP Stabilizer and Biostab at 100ng/ml concentration of detection antibody. The antigen concentration is maintained at 2.5ug/ml.

		In House AP Stabilizer Buffer		Stabilzyme		Biostab	
Date	Sample Type	Mean RLU	CV%	Mean RLU	CV%	Mean RLU	CV%
1/20/2012	USBiol Kit Pos CTL	61510	6.4	47429	12.3	163508	23.3
	Biochain Kit Pos CTL	51609	22.8	34414	12.8	128808	13.4
	Abnova Kit Pos CTL	30540	15.5	18336	13.4	74300	10.9
	Mean Positive	47886		33393		122205	
	Pooled Negative Neg CTL	2560	18.9	1185	9.0	6900	19.4
Mod b/w Mean Pos CTL and Neg CTL		18.7		28.2		17.7	

3 BACK-UP ANTIGEN DEVELOPMENT

Antigen 6 was used as a back-up antigen in the event that Antigen 2 is out of stock. Antigen 6 demonstrated good modulation and specificity. A general cutoff value for the Theranos system was determined by taking the mean RLU of the normal samples plus 2.5 times the standard deviation of the normal samples.