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Vitamin B12
Assay Development 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 detect vitamin B12 in human serum and plasma. The assay has a reportable range of 250 to 4000 pg/mL. The Theranos serum B12 assay is calibrated using the Cyanocobalamin, meets USP testing specifications from (Cat#C3607-500MG) from Sigma.

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

The reference assay is the Vitamin B12 assay on the Siemens Immulite 2000 from the CLIA lab.

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

Avidin serves as the capture surface. The sample is treated sample treatment buffers to release the vitamin B12 bound to endogenous binding proteins for 10 min, neutralized, combined with biotin labeled pig intrinsic factor binding protein and the tracer, alkaline phosphatase labeled B12. This mixture is incubated on the capture surface for 10 minutes. After the incubation, the surface is washed and substrate is incubated on the surface for 10 minutes, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]; Materials

Name	Supplier	Catalog #
Cyanocobalamin	Sigma	C3607-500MG
Alkaline Phosphatase Substrate	Theranos	T-ALKP-SB01
Albumin, Human Serum (HSA) Low B12/Low Folate	Meridian	H8P01-767
UltraAvidin	Leinco Technologies	A110
Intrinsic factor (binding protein)	Scripps Labs	I1024
0.1 N HCL	BDH	BDH3200-1
0.2 N NaOH	BDH	BDH3220-1
KCN	Sigma	60178-25G
Dicyanocobinamide	Sigma	C3021-10MG
DTT	Sigma	646563-10X.5ML
EDTA	Sigma	03690-100ML
Mannitol	Sigma	63560-250G-F
B12-GA-AP conjugate	Theranos	
Carbonate-bicarbonate buffer	Sigma	C3041
Serum substitute supplement	Irvine Scientific	99193

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

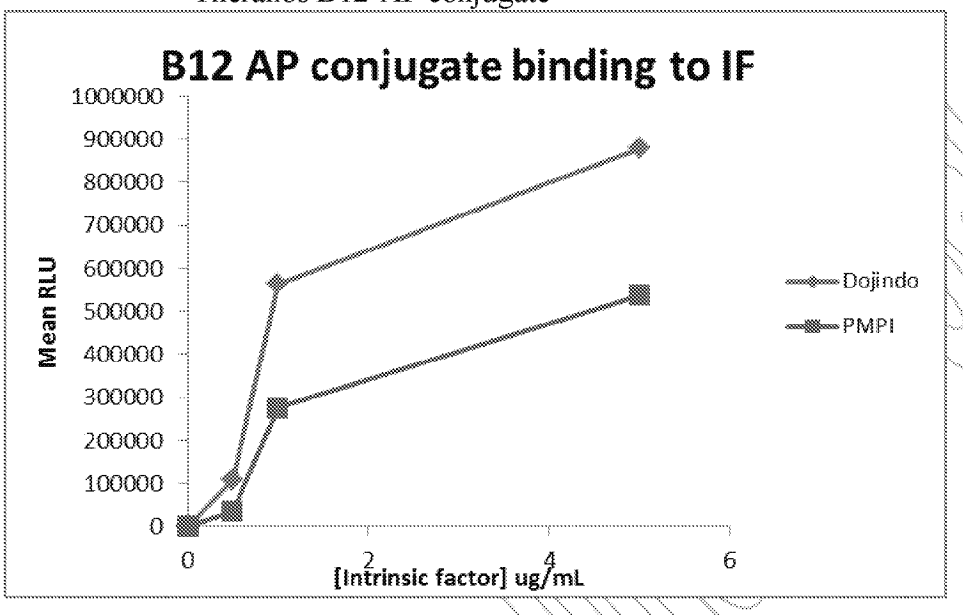
1.2 Conjugate Binding to Intrinsic Factor binding protein (MTP) [TC "Detection Antibody Conjugate Verification" \f C \l "1"]

The B12 binding protein , intrinsic factor, binds to both the Dojindo and the Theranos B12 AP conjugates. A pH dependent study was also carried out wherein the conjugates were in two different stabilizers at pH 8.0 and pH 9.0. Table 2 summarizes the data.

Table [SEQ Table * ARABIC]: Antibody-Conjugate Binding Screen with Dojindo and Theranos B12-AP conjugate

Dojindo B12 AP Conjugate, 100 ng/ml								
	[IF] ug/mL	Mean	CV%	S/B		[IF] ug/mL	Mean	CV%	S/B
pH 8.0	5	879121	8	1526	pH 9.0	5	637192	2.6	1370
	1	564451	15.4	980		1	355443	7.3	764
	0.5	109085	4.3	189		0.5	54648	27.2	118
	0	576	21.9	1		0	465	13.5	1
Theranos B12-PMPI- AP Conjugate, 1:10,000 dilution									
	[IF] ug/mL	Mean	CV%	S/B		[IF] ug/mL	Mean	CV%	S/B
pH 8.0	5	537494	18.7	507	pH 9.0	5	380634	1.5	430
	1	276398	2.3	261		1	192587	2.6	217
	0.5	34968	1	33		0.5	19923	21.6	22
	0	1061	6.3	1		0	886	7.3	1

Figure [SEQ Figure * ARABIC]: Antibody-Conjugate Binding Screen with Dojindo and Theranos B12-AP conjugate



1.3 Competitive Assay Screen (MTP)

The tracers from Theranos (B12 AP conjugate) and Monobind kit (B12 HRP conjugate) were tested in the following competitive assay format: the surface was coated with 0.5 ug/ml of the binding protein, sample treatment reagents from the Monobind kit were used. The assay format was 10' incubation of the sample followed by 10' incubation of the tracer followed by substrate incubation at 10 min. The data are summarized in Table 3 and 4. The Monobind tracer provided better dose response for both the calibrators and clinicals compared to the Theranos B12 tracer when tested on the Theranos coating surface. This provided an idea that the surface was working and the key optimization strategies would lie in the sample treatment steps.

Table [SEQ Table * ARABIC]: Competitive Assay with B12 Monobind HRP conjugate

Conc pg/mL	Theranos Calibrators	Clinicals	Siemens data , pg/mL	Sample ID
2000	656	1432	2121	B23
1000	1225	1111	1156	B24
500	2154	2282	471	B9
250	2927	2899	241	B54
100	3827	3193	165	B3
0	4085	3908	159	B4
S/B	6.2	3.5	H/L	

Table [SEQ Table * ARABIC]: Competitive Assay with B12 Theranos AP conjugate

Conc pg/mL	Theranos Calibrators	Clinicals	Siemens data pg/mL	Sample ID
2000	113810	130356	2121	B23
1000	126566	90315	1156	B24
500	142200	167964	471	B9
250	152698	125051	241	B54
100	169036	187747	165	B3
0	180415	182098	159	B4
S/B	1.6	2.0	H/L	

1.4 On Board Sample Treatment using Monobind Kit Sample treatment Reagents

The following assay format used to test the B12 competitive assay on the Theranos 3.0 system: avidin on the surface, intrinsic factor binding protein labeled with biotin in solution. Free B12 after release from sample following treatment) would compete with labeled tracer B12-AP conjugate for binding to the B12 binding protein. A standard curve of B12 calibrators (spiked into a serum substiutute material) was used as calibration. A small set of clinical samples were tested. The sample treatment reagents used for this experiment still came from the Monobind kit. The results are summarized in Table 5 and Table 6 and in Figure 2. The standard curve data showed a signal to background of 15. Figure 2 shows the clinical correlation of the small set of clinicxal samples tested top the Siemens Immulite results. The correlation was promising R value of 0.97 and slope of 1.1. This preliminary data demonstrated that the sample treatment procedure as well as the assay format was working.

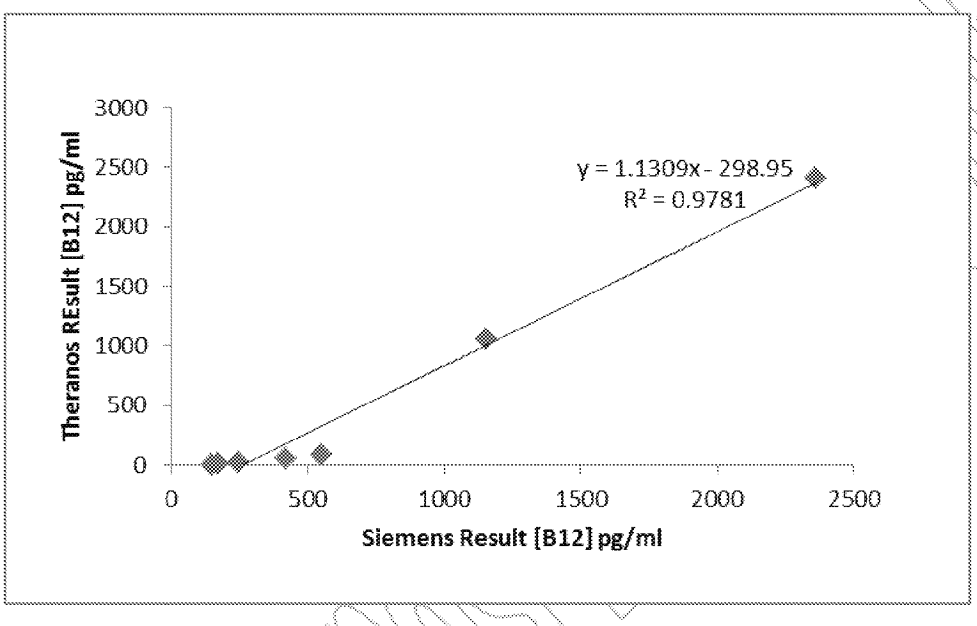
Table [SEQ Table * ARABIC]: Standard Curve : Spiked B12 in serum substitute reagent

[B12] pg/ml	Inter-Cartridge Mean	CV%	Log S	Log C	Inter-Cartridge Mean	CV%	% Recovery	S/B
2000.0	10668	1	4.028	3.30	2040	0.9	102	15
1000	28133	3	4.449	3.00	1033	2.2	103	6
500.0	70612	4	4.849	2.70	477	18.4	95	2.3
250	83984	2	4.924	2.40	285	10.3	114	1.9
100.0	107142	4	5.030	2.00	100	38.4	100	1.5
0	159275	4	5.202		7	9.0		1.0

Table [SEQ Table * ARABIC]: Clinical samples: Comparison to Results on Siemens Immulite

ClinicaL Sample	Siemens Result pg/mL	Inter-Cartridge Mean	CV%	Log S	Inter-Cartridge Mean	CV%
B22	>1000	8207	17	3.914	4511	12.0
B24	2361	9977	5	3.999	2407	23.0
B34	1156	19105	4	4.281	1053	16.0
B11	548	111583	12	5.048	81	8.0
B51	421	122162	2	5.087	48	20.0
B53	248	137099	8	5.137	22	12.0
B2	171	149789	38	5.175	11	28
B1	150	178600	1	5.252	2	19
	H/L	**21.8**				

Figure [SEQ Figure * ARABIC]: Clinical Correlation: Theranos vs. Siemens: Sample treatment on the Theranos 3.0 using Monobind kit Reagents



1.5 Testing formulations for Sample treatment Reagents

The industry standard protocol for the treatment of a clinical sample prior to testing on the B12 assay comprises the following steps: (i) denaturation using a high pH buffer (this step aims to release B12 bound to binding proteins in the sample by denaturing them) (ii) treatment with a reducing agent to further breakdown binding proteins as well as intrinsic factor autoantibodies, (iii) including KCN in the formulation (to convert all forms of cobalamin to cyanocobalamin) and (iv) inclusion of dicyanocobinamide, an analog of cobalamin (to saturate all the R proteins that escaped denaturation) (v) neutralization of sample prior to binding to binding protein or anti-B12 antibody. The formulations of the high pH buffer and reducing agents were arrived at by repeated testing with different normalities of the NaOH and titrating different concentrations of the reducing agent dithiothreitol, DTT. The assay was carried out as previously on the Theranos system with avidin surface and the biotin labeled binding protein in solution. Free B12 released from the sample competed with the labeled tracer, B12 AP for binding to the intrinsic factor binding protein. For the purposes of this experiment a top level and zero calibrator were used and the signal to background ratio was used to determine which formulations worked the best. Table 7 summarizes the results of the various formulations tested. As can be seen from the results Theranos high pH and DTT buffer formulations were successfully optimized but the various buffers tested as neutralization buffers did not produce any modulation. At this point in the optimization only the best signal to background ratio was only afforded when the Monobind reagent was used as the neutralization buffer.

Table [SEQ Table * ARABIC]: Clinical samples: Comparison to Results on Siemens Immulite

Monobind high pH	√		√	√	√	√	√	√	√	
Monobind DTT	√	√		√	√					
MonobindB neutralization	√	√	√							√
Theranos high pH		√								√
Theranos DTT			√			√	√	√	√	√
Theranos neutralization										
Low HAS/TBS pH 8.0						√				
Phosphate citrate buffer pH 6.5								√		
Low HAS/borate pH 9.0									√	
20 mM phosphate pH 5.8										
100 mM citrate pH 5.6				√	√					
Sea Block							√			
S/B	**15**	**4**	**32**	**1**	**1**	**1**	**1**	**1**	**1**	**22**

1.1 Evaluation of Assay Formats

More changes in the on board sample treatment protocol were tested in order to remove the dependence on a commercial reagent (Monobind neutralization buffer). These included providing separate locations for the neutralization buffer and binding protein in the cartridge at the same time maintaining the effective sample dilution at 5-fold. This new protocol provided a good platform to include testing of two more assay formats as well as testing additional neutralization buffer formulations. The formats differed in the presentation of the binding protein.

Table [SEQ Table * ARABIC]: Assay format testing

| SHAPE * MERGEFORMAT |

Format 1 shown as the first entry in Table 8 included an anti-intrinsic factor clone as the surface. The unlabeled intrinsic factor binding protein was presented in solution. Free B12 released from sample competed with the B12-AP tracer for binding to the intrinsic factor protein. This format gave the best modulation between the top level and zero calibrator and performed well across the different high pH solutions and neutralization buffers tested. The second format was the format with avidin on the surface and the biotin labeled binding protein in solution. This was the next best format in terms of the overall modulation. A third format tested included the B12 BSA conjugate coated surface. The biotin labeled intrinsic factor binding protein was presented in solution. Competition between the free B12 released from sample and the B12 BSA tracer would drive the dose response. The detection system was a avidin labeled alkaline phosphatase conjugate that would bind to the biotin labeled binding protein. Format 2 was finalized as the final assay format and format 1 and 3 could be potential back ups.

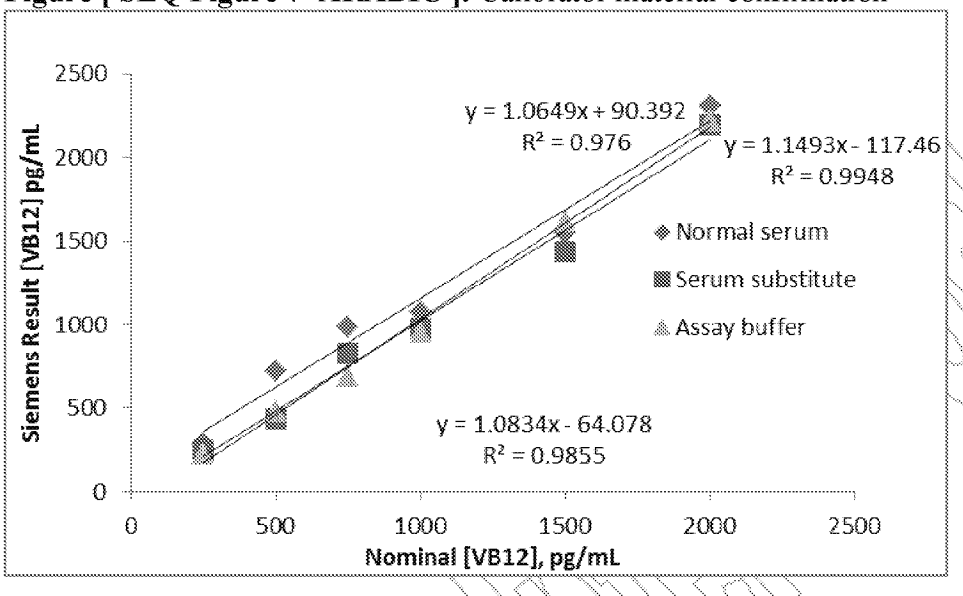
1.2 Calibrator material confirmation

The vitamin B12 analyte used as calibration material was obtained from Sigma and certified to have met USP testing specifications. This material was spiked into normal serum, serum substitute and assay buffer and verified on the Siemens Immulite Vitamin B12 assay. As the results summarized in Table 9 show there was very good correlation between the nominal calibrator concentrations and the reported values from the Siemens assay. This served as a confirmation of the calibration material.

Table [SEQ Table * ARABIC]: Calibration material confirmation.

Normal serum		Serum substitute		Assay buffer	
CLIA Results	Nominal	CLIA Results	Nominal	CLIA Results	Nominal
pg/mL		pg/mL		pg/mL	
2313	2000	2195	2000	2217	2000
1547	1500	1434	1500	1620	1500
1075	1000	976	1000	954	1000
987	750	830	750	689	750
723	500	434	500	483	500
287	250	247	250	228	250
< 150	100	< 150	100	< 150	100
< 150	0	< 150	0	< 150	0

Figure [SEQ Figure * ARABIC]: Calibrator material confirmation



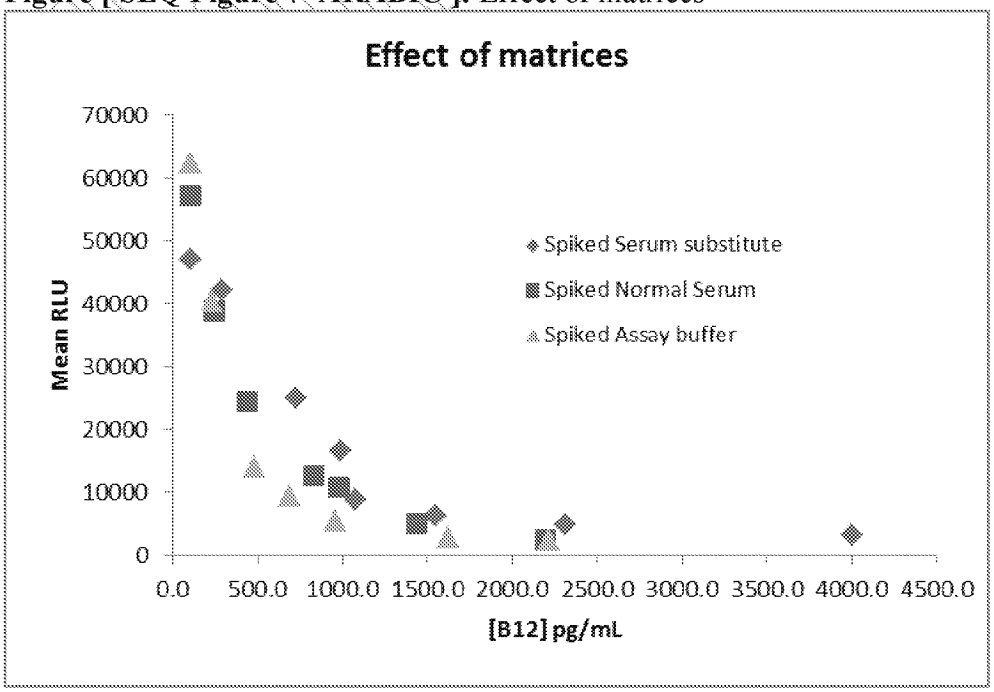
1.3 Effect of matrices: Choice of matrix for calibration

The calibrators made and tested on the Siemens Immulite assay as described in the previous section were tested on the Theranos B12 assay in order to compare the dose response of the assay in different matrices. The data are summarized in Table 10 and Figure 4. The dose response is identical in all three matrices. It was decided to finalize serum substitute as the calibration matrix since B12 depleted serum was not commercially available and it was difficult to obtain larger volumes of sera that had low endogenous levels of vitamin B12.

Table [SEQ Table * ARABIC]: Effect of matrices: choice of calibration matrix

	Spiked Serum substitute				Spiked Normal Serum				Spiked Assay buffer			
Nominal [B12] pg/mL	Siemens [B12] pg/mL	Inter-Cartridge Mean	Inter-Cartridge CV%	S/B	Siemens [B12] pg/mL	Inter-Cartridge Mean	Inter-Cartridge CV%	S/B	Siemens [B12] pg/mL	Inter-Cartridge Mean	Inter-Cartridge CV%	S/B
4000	4000.0	3303	8	20.8								
2000.0	2313	4887	4	14.1	2195	2497	15	22.1	2217	2384	1	25.4
1500	1547.0	6284	4	11.0	1434.0	5087	16	10.9	1620.0	2965	16	20.5
1000.0	1075	8972	7	7.7	976	10880	12	5.1	954	5557	7	10.9
750	987.0	16626	6	4.1	830.0	12614	9	4.4	689.0	9432	5	6.4
500.0	723	25008	5	2.8	434	24442	7	2.3	483	14101	21	4.3
250	287	42176	9	1.6	247	38913	17	1.4	228	40424	26	1.5
100	100	47099	12	1.5	100	57216	18	1.0	100	62383	20	1.0
0	0	68860	1	1.0	0	55272	12	1.0	0	60666	24	1.0

Figure [SEQ Figure * ARABIC]: Effect of matrices



1.4 Cross reactivity and Interference

Cross reactivity against dicyanocobinamide which is an analog of cyanocobalamin was tested by assaying different levels ranging from 20-1 ug/mL. All the levels tested showed out of range low values for vitamin B12 confirming that there was no detectable cross reactivity with this analog (Table 11). Dicyanocobinamide at an excess concentration of 60 ug/mL was spiked into the vitamin B12 calibration curve and the back calculated concentrations were compared to control standard curve. All recoewvries were within 20% of nominal (Table 12) indicating that there was no interference from this analog on the Theranos vitamin B12 assay.

Table [SEQ Table * ARABIC]: Cross reactivity with dicyanocobinamide

(CN)2Cbi ug/ml	Inter-Cartridge		Theranos
	Mean	CV%	Result
20	76234	8	OORL
10	67238	3	OORL
5	62001	12	OORL
2.5	66626	7	OORL
1	62312	25	OORL
0	57412	12	OORL

OORL=out of range low

Table [SEQ Table * ARABIC]: Interference with dicyanocobinamide

Nominal [B12]pg/mL	Inter-Cartridge		S/B	% Recovery from Ctrl
	Mean	CV%		
4000	3600	3	19.1	109
2000	5518	2	12.5	113
500	18825	11	3.7	75
250	36506	72	1.9	87
100	55113	5	1.2	117
0	57596	3	1.2	84

1.5 Training Set

In order to confirm format 2 as the final format a small set of clinical samples were tested on the assay with the current on board conditions for sample treatment. The calibration curve was based on vitamin B12 spiked serum substitute material described in section 1.3. Table 13 summarizes the calibration curve parameters and Figure 5 depicts the curve fit for this calibration. Using this calibration, 10 clinical samples across the range were tested and the back calculated result was compared to the values reported by the Siemens Immulite vitamin B12 assay. This data is summarized on Table 14 and Figure 6. As can be seen there was good correlation between the Theranos result and the Siemens Immulite data (R value of 0.98 and slope of 0.80).

Table [SEQ Table * ARABIC]: Calibration curve parameters

ULOQ	4000	pg/mL
LLOQ	250	pg/mL
ULOQ accuracy	119	%
ULOQ precision	14.5	%
LLOQ accuracy	100	%
LLOQ precision	14.3	%

Figure [SEQ Figure * ARABIC]: Calibration curve

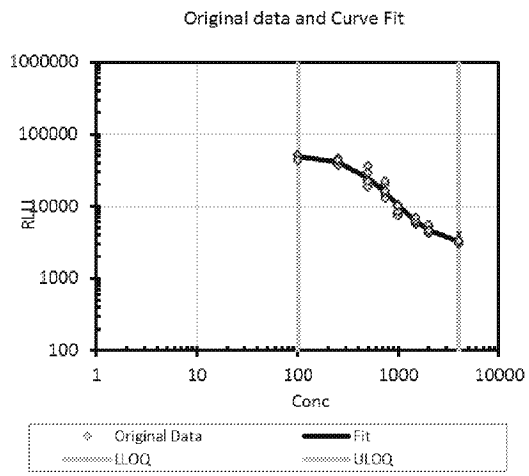
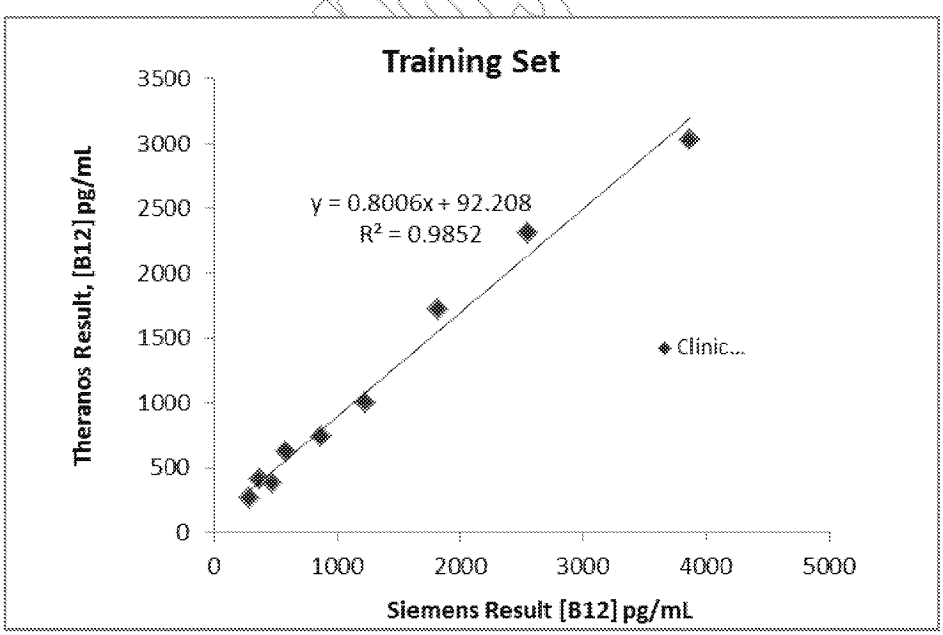


Table [SEQ Table * ARABIC]: Clinical Correlation: Theranos vs. Siemens: Training Set

Sample ID	Siemens B12 result pg/mL	Inter-Cartridge RLU Mean	Inter-Cartridge RLU CV%	Backcalculated Conc. Mean	Backcalculated Conc. %Cv
B28	2547	4245	8	2314	15
B31	1231	8518	2	1002	16
B14	582	21169	3	623	12
B32	1825	5718	2	1723	9
B15	871	29137	19	736	7
B16	374	37748	4	412	17
B9	471	35229	16	381	18
B31	3867	5287	8	3027	12
B37	285	43081	3	261	28
B4	159	67887	5	OORL	21

Figure [SEQ Figure * ARABIC]: Clinical Correlation: Theranos vs. Siemens: Training Set



1.6 Checkerboard titration of Binder and Tracer

Both the binder and the tracer were titrated to arrive at conditions that would provide the highest modulation. The different conditions were tested with B12 spiked serum substitute calibrators. The highest S/B and modulation at the low end were seen at the binding protein concentration of 0.05 ug/ml and at the tracer diluted 1:25,000. These were finalized.

Table [SEQ Table * ARABIC]: Checkerboard titration

	[Tracer]	1:6250				1:12500				1:25,000			
[B12] pg/ml	**[IF-BT] in soln ug/mL**	**Inter-Cartridge Mean**	**CV%**	**S/B**	**Mod.**	**Inter-Cartridge Mean**	**CV%**	**S/B**	**Mod.**	**Inter-Cartridge Mean**	**CV%**	**S/B**	**Mod.**
4000.0		1719	0	24	4.4	2760	6	15	2.4				
1000	0.025	7511	12	6	4.6	6753	9	6	5.5		nd		
250.0		34901	2	1.2	1.2	37384	37	1.1	1.1				
0		41704	20	1		42240	16	1					
4000.0		3321	21	22	2.1	2608	14	29	2.9	1794	6	**37**	6.6
1000	0.05	7079	1	11	9.1	7515	2	10	9.3	11880	9	6	4.5
250.0		64297	2	1.2	1.2	69799	11	1.1	1.1	53161	20	1.3	1.3
0		74547	38	1		75809	9	1		67228	2	1	
4000.0		nd				3937	33	22	2.4	2339	33	41	3.9
1000	0.1					9261	37	9	8.8	9211	37	10	9.0
250.0						81234	2	1.1	1.1	82981	2	1.2	1.2
0						86470	4	1		96470	4	1	

1.7 Effect of AP conjugate stabilizer

The B12 AP conjugate was formulated in 3 different AP conjugate stabilizer and the dose resposne was evaluated as shown in Table 15. The Theranos Small Moleculae AP Conjugate Stabilizer showed the best modulation between the different calibrator levels and the best overall S/B. It was finalized as the AP conjugate stabilizer.

Table [SEQ Table * ARABIC]: Effect of AP conjugate stabilizer

	Theranos				Biostab				StabilZyme Noble			
[B12] pg/ml	Inter-Cartridge Mean	CV%	S/B	Mod.	Inter-Cartridge Mean	CV%	S/B	Mod.	Inter-Cartridge Mean	CV%	S/B	Mod.
4000	1719	11	32	2.7	2760	6	15	1.9	3296	18	19	1.3
2000	4712	12	12	1.6	5123	16	8	1.3	4423	34	14	1.4
1000	7511	21	7	3.3	6753	9	6	3.4	6123	23	10	2.4
500	25132	16	2.2	1.5	23156	18	2	1.6	14512	17	4	3.1
250	36901	19	1.5	1.5	37384	37	1	1.1	45612	16	1.4	1.4
0	55704	20	1	0	42240	16	1	0	62340	13	1	0

1.8 Effect of Sample Diluent

To determine if various blocking buffers might improve the assay when used as sample diluents, 2 commercial blockers were tested against the Low BSA buffer. The commercial blockers further lowered the modulation so it was decided to finalize the low BSA blocking buffer (0.03% BSA/0.05% NaN_3 in TBS) as the sample diluent. Data are summarized in Table 17.

Table [SEQ Table * ARABIC]: Effect of Sample diluent

	Control				Super Block			
[B12] pg/ml	Inter Cartridge		S/B	Mod.	Inter Cartridge		S/B	Mod.
	Mean	CV%			Mean	CV%		
4000	1719	11	32	2.7	2806	4	15	1.8
2000	4712	12	12	1.6	5123	21	8	1.3
1000	7511	21	7	3.3	6753	14	6	3.4
500	25132	16	2.2	1.5	23156	25	1.8	1.5
250	36901	19	1.5	1.5	34119	19	1.2	1.2
0	55704	20	1	0	41671	13	1	0
	Low Cross				Surmodics			
[B12] pg/ml	Inter-Cartridge		S/B	Mod.	Inter-Cartridge		S/B	Mod.
	Mean	CV%			Mean	CV%		
4000	2452	13	18	1.8	2198	19	27	2.3
2000	4423	12	10	1.4	4989	12	12	1.6
1000	6123	9	7	5.8	8231	34	7	3.9
500	35671	14	1.3	0.9	32154	12	1.9	1.4
250	31546	32	1.4	1.4	45612	21	1.3	1.3
0	44759	13	1	0	59518	12	1	0

1.9 Compare In house versus Commercial Alkaline phosphatase substrate

The dose response for the vitamin B12 assay was tested with the newly available in house formulated AP substrate. The response was compared to the commercial substrate from KPL. The in house substrate seemed to reduce the background considerable thereby boosting the S/B for the assay (Table 18). The in house substrate was adopted for all remaining assay development experiments.

Table [SEQ Table * ARABIC]: Compare In house versus Commercial AP substrate

	KPL Substrate			Theranos Substrate		
Nominal B12 pg/mL	**Inter-Cartridge**			**Inter-Cartridge**		
	Mean	**CV%**	**S/B**	**Mean**	**CV%**	**S/B**
4000	1725	11	39.2	466	34	149.5
2000	4392	13	13.1	2734	11	25.5
1500	4946	14	11.7	3777	36	18.5
1000	8223	8	7	6734	13	10.4
500	23380	15	2.5	26222	5	2.7
250	36926	23	1.6	42248	8	1.7
100	36464	15	1.6	44285	39	1.6
0	67646	13	1	69739	14	1

1.10 Test other versions of the B12 tracer

Two other versions of the B12-AP conjugate were prepared in house and tested against the control tracer. Each tracer differed in the length of the spacer arm of the cross linker molecule that linked the cyanocobalamin to the alkaline phosphatase enzyme. The three tracers were tested side by side to see if this affected the dose response and sensitivity of the assay. It was observed that the longer the spacer arm length the higher the signal to background reached (Table 19). However the sensitivity at the low end did not improve with the change in the tracer. For this reason the control tracer (B12-GA-AP, spacer arm length of cross linker = 17 Å) was finalized as the tracer to be used in the Theranos vitamin B12 assay.

Table [SEQ Table * ARABIC]: Compare B12-AP conjugates

	17 Å			30 Å			8.7 Å		
	B12-GA-AP			B12-PEG-AP			B12-PMPI-AP		
Nominal B12 pg/mL	Inter-Cartridge Mean	CV%	S/B	Inter-Cartridge Mean	CV%	S/B	Inter-Cartridge Mean	CV%	S/B
4000	478	5	124	439	12	146	503	24	41
1000	4670	2	13	12997	1	5	3848	9	5
500	13612	10	4.4	31825	26	2	8259	8	2.5
250	24666	14	2.4	49793	2	1.3	14973	27	1.4
100	32698	2	1.8	50609	13	1.3	15966	1	1.3
0	59459	1	1	64263	3	1	20737	8	1

1.11 Final Calibration

With all the assay conditions finalized so far a new lot of reagents were made and a new calibration curve was produced using these regents. The ULOQ for the assay was 4782 pg/mL and the LLOQ was 266 pg/mL. The calibration curve is summarized in Table 20 and the parameters for accuracy and precision are poutlined in Table 21.

Table [SEQ Table * ARABIC]: Determination of LLOQ and ULOQ

Conc = b3 * (((b2 - b1) / (log10(RLU) - b1)) - 1) ^ (1 / b4)

Nominal B12 pg/mL	Assigned Siemens	RLU Mean	RLU CV%	S/B	Conc, pg/ml Mean	Conc, pg/ml CV%	% Recovery
4000	4782.0	415	4	111	4534	6.0	95
2000	2313.0	820	24	56	2317	14.7	100
1500	1547.0	1514	24	30	1615	11.9	104
1000.0	987	5135	18	9	932	8.1	94
500	637	12362	7	4	605	3.8	95
250	266	29726	13	1.5	289	19.2	109
155	155	32677	11	1.4	246	21.7	159
100	100	35335	5	1.3	207	12.6	207
0	0	46034	8	1	OORL		

Conc = 28.54* (((394776.9-10004.8) / (RLU -10004.8)) - 1) ^ (1 /1.242)

Table [SEQ Table * ARABIC]: Calibration Parameters

LLOQ	**266.00**	**pg/mL**
ULOQ	**4782.00**	**pg/mL**
LLOQ accuracy	**102**	**%**
LLOQ precision	**10.0**	**%**
ULOQ accuracy	**107**	**%**
ULOQ precision	**19.3**	**%**

1.12 Calibration Verification

The same lot of reagents was used for the remainder of the assay development so the calibration equation from Table 20 was used for back calculating B12 concentrations in samples in the next experiments. Commercially available control materials with reported values for B12 were tested to verify assay calibration. These included the WHO/NIBSC international standard 03/178, the Non-WHO Pernicious anemia control and other controls from BioRad. Also included in the testing were anti –intrinsic factor antibody controls from an FDA-approved anti-intrinsic factor antibody assay. The data are summarized in Table 22. The recoveries of the Bio Rad and WHO controls were within 20% of reported value. The anti-IF antibodies and pernicious anemia controls were not detected by the assay.

Table [SEQ Table * ARABIC]: **Calibration Verification**

Controls	Siemens	RLU		Conc. pg/ml		% Recovery
	Result B12 pg/mL	Mean	CV%	Mean	CV%	
Biorad Liquichek level 1	208	41746	18	236	10.5	113
Biorad Liquichek level 2	499	28594	17	381	10	76
Biorad Liquichek level 3	840	7437	14	713	8	85
BioRad lyphocheck 1	332	50434	7	237	3	71
BioRad lyphocheck 2	581	29199	28	435	14.9	75
BioRad lyphocheck 3	1500	2136	16	1510	10.3	101
WHO/NIBSC/03/178	480	28032	18	457	7.8	95
Non -WHO Pernicious anemia	<150	49823	17	OORL		
BioRad Anemia Control	155	32764	12	OORL		
Anti-IF Ab controls 1	<150	43467	13	OORL		
Anti-IF Ab controls 2	<150	48234	10	OORL		
Anti-IF Ab controls 3	<150	48123	23	OORL		

1.13 Interference Test for RF and HAMA positive samples

The Theranos B12 assay was tested for interference from RF positive and HAMA positive samples. 5 samples of each type were tested on the Theranos system as well as the CLIA lab B12 assay. The recoveries for all 10 samples were within 20% of the reported value from Siemens assay indicating that there was no interference from these sample types on the assay.

Table [SEQ Table * ARABIC]: **RF positive sample testing**

Sample Id	RLU		Conc. pg/ml		Siemens Result	% Recovery
	Mean	CV%	Mean	CV%	ng/ml	
R1	20313	9	433	7.2	370	117
R2	31073	12	268	20.3	302	89
R4	18662	15	466	10.6	410	114
R5	5702	11	886	4.7	894	99
R7	12880	2	589	1.1	618	95

Table [SEQ Table * ARABIC]: **HAMA positive sample testing**

Sample Id	RLU		Conc. pg/ml		Siemens Result	% Recovery
	Mean	CV%	Mean	CV%	ng/ml	
H1	27346	17	323	21.2	370	87
H2	26504	28	341	32.9	310	110
H3	17066	20	497	13.4	560	89
H4	13332	24	584	13.0	771	76
H5	9055	17	718	8.6	639	112

1.14 Interfering Matrices

Hemolyzed, lipemic and icteric serum samples were obtained from a commercial source. The recovery of B12 spiked into these potentially interfering matrices was evaluated on the Theranos System. The assay did not show any interference from hemolyzed and icteric samples judging from the recoveries which were within 20% of nominal (Table 25). The assay showed only about 70% recovery for the lipemic sample tested.

Table [SEQ Table * ARABIC]: **Effect of interfering matrices**

	Lipemic					Hemolytic					Icteric				
Nominal	Mean RLU		Conc. pg/ml		% Recovery accounting for endogenous	Mean RLU		Conc. pg/ml		% Recovery accounting for endogenous	Mean RLU		Conc. pg/ml		% Recovery accounting for endogenous
	Mean	CV%	Mean	CV%		Mean	CV%	Mean	CV%		Mean	CV%	Mean	CV%	
2000	1108	26	1939	14.2	86	750	10	2432	8.0	110	625	6	2808	5.3	115
1500	1918	10	1436	4.1	82	1411	2	1651	1.0	96	902	6	2156	4.5	111
1000	4717	1	974	0.7	77	5443	9	906	3.8	74	1752	5	1487	2.3	104
500.0	15493	11	527	7.0	69	10186	4	670	2.3	93	6243	9	850	4.4	91
250	27358	1	322	0.7	63	23958	21	376	20.6	80					
0	31644	5	261	8.2		35105	6	219	18.1		20311	13	435	10.8	

This indicated that grossly lipemic sample types should be avoided as they interfere with the assay.

1.15 Matched Sample Analysis

5 whole blood samples from male donors and 5 from female donors were collected from a blood bank. Each donor provided blood that was collected in K2EDTA, Li-heparin and serum separation containers. Each of the above sample was spun down to generate plasma/serum and sent to the CLIA lab to obtain results on the Siemens Immulite vitamin B12 assay. Each sample was also tested on the Theranos B12 assay. The data are summarized in Table 26.

Table [SEQ Table * ARABIC]: Matched sample analysis

	EDTA plasma		Li-Heparin plasma		Serum	
Sample Id	Siemens Result pg/mL	Theranos result pg/mL	Siemens Result pg/mL	Theranos result pg/mL	Siemens Result pg/mL	Theranos result pg/mL
ME1	887	822	821	879	761	860
ME2	645	614	600	637	598	656
ME3	903	1028	850	1044	877	878
ME4	755	706	752	699	741	707
ME5	1235	1165	1077	1051	1086	1007
FE1	368	401	322	330	332	368
FE2	398	366	379	342	487	343
FE3	367	369	340	342	359	346
FE4	191	OORL	190	OORL	184	OORL
FE5	726	620	711	523	770	506

The results correlated excellently with the Siemens data and showed that there is no effect of the different anticoagulants on the assay. Figures 7-11 depict the correlation plots and the slopes therein confirm that the recoveries were within 20% of the reported value from Siemens.

Figure [SEQ Figure * ARABIC]: EDTA plasma: Theranos vs. Siemens

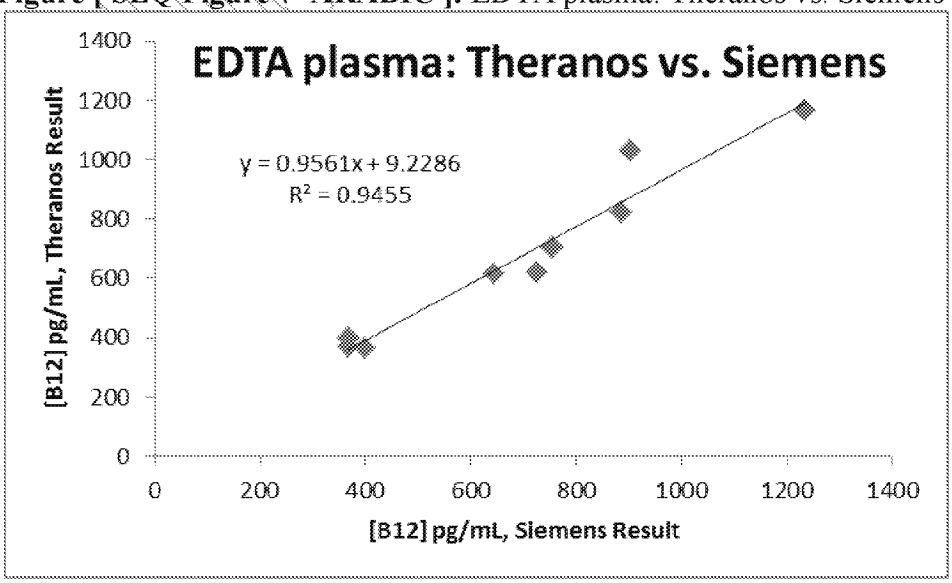


Figure [SEQ Figure * ARABIC]: Li Heparin plasma: Theranos versus Siemens

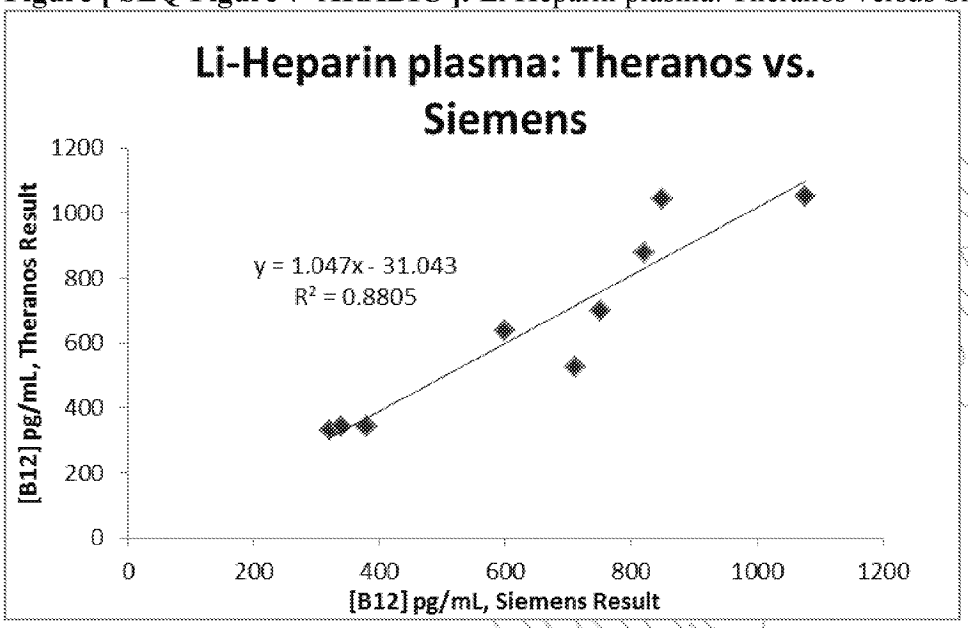


Figure [SEQ Figure * ARABIC]: Serum: Theranos versus Siemens

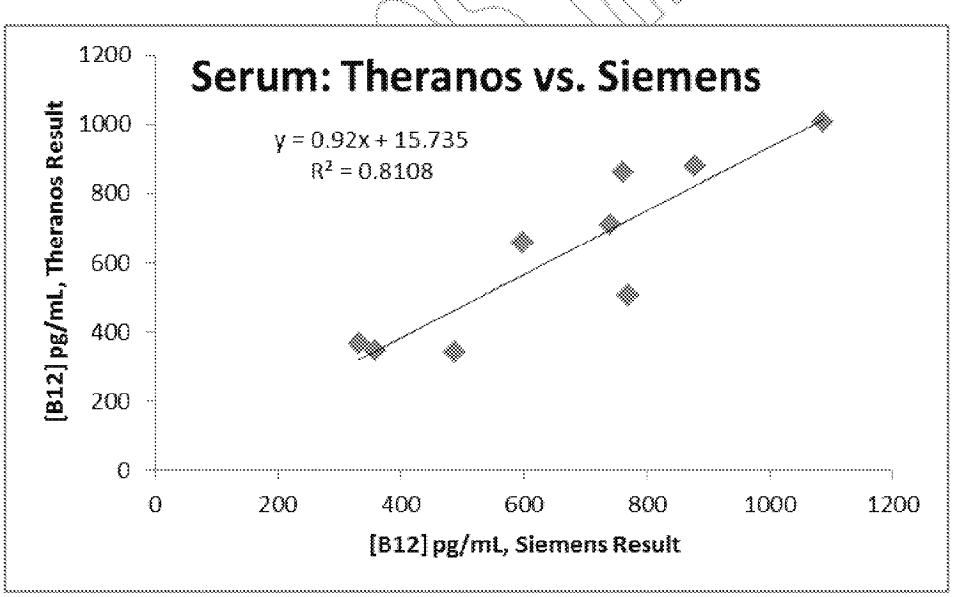


Figure [SEQ Figure * ARABIC]: EDTA plasma vs. serum: Theranos

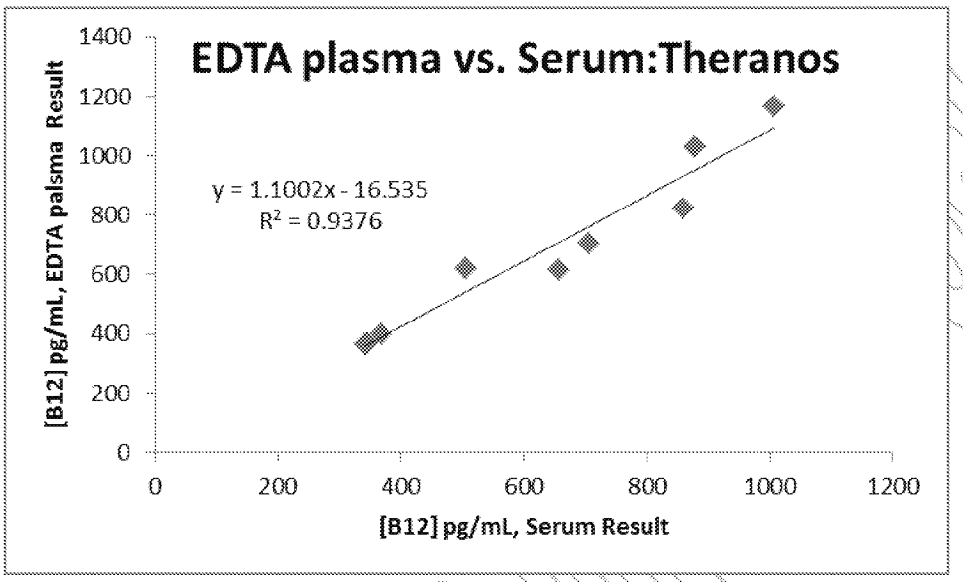
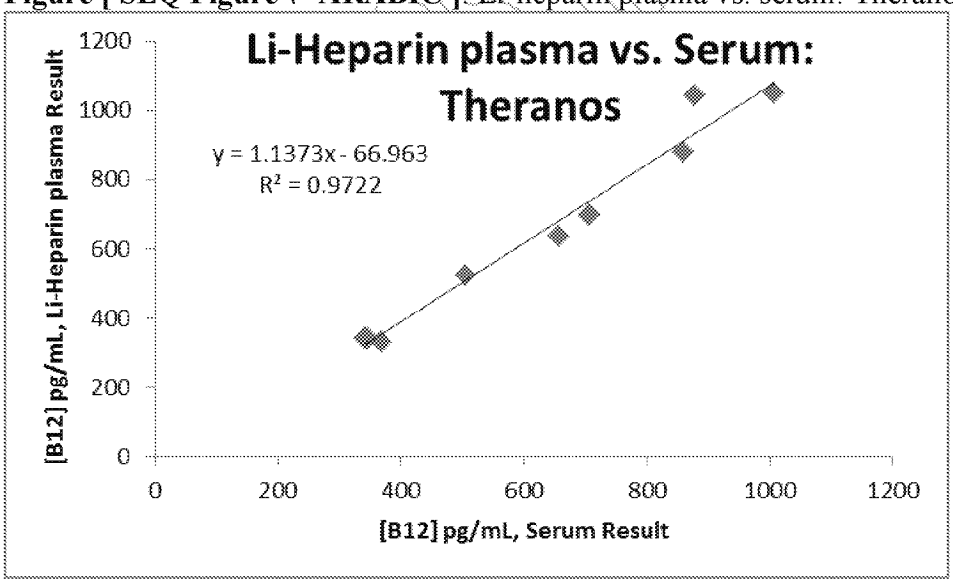


Figure [SEQ Figure * ARABIC]: Li-heparin plasma vs. serum: Theranos



1.16 Clinical Sample Correlation

20 serum samples from anemic subjects and 14 from clinical subjects were obtained from commercial sources. All samples were also run on the Siemens immulite B12 assay. The anemic subjects turned out to have high levels of B12. This was confirmed by looking at the patient information sheets obtained from the commercial vendors which cited the patients taking vitamin B12 supplements. The correlation with the CLIA assay was excellent with an R value of 0.96 and a slope of 0.82. Data summarized on Table 27.

Table [SEQ Table * ARABIC]: Clinical Sample Correlation

Sample Id	RLU		Theranos Conc. pg/mL		Siemens Results
	Mean	CV%	Mean	CV%	pg/ml
Ane1	4742	11	959	5	961
Ane2	27305	15	324	19	676
ane3	22453	9	397	8	361
Ane4	24684	4	366	5	498
Ane5	4763	14	967	7	1020
Ane6	10059	35	695	19	942
Anc7	18244	24	480	18	773
Ane8	17419	18	489	13	680
Ane9	738	4	2505	3	2884
Ane10	19148	15	460	13	766
ane 11	13716	18	573	11	935
Ane 12	28460	21	307	29	590
ane 13	31420	6	264	10	233
ane14	23975	11	374	11	556
Ane 15	24111	25	377	26	518
Ane16	11376	22	638	12	743
ane 17	18169	31	485	24	737
ane 18	1575	4	1568	2	1451
Ane 19	15933	6	517	4	477
Ane 20	14414	7	555	4	736
B56	38223	19	OORL		193
B57	10902	13	648	7.3	822
B58	918	15	2143	8.5	2721
B59	32851	4	243	7.8	234
B60	25889	13	345	14.9	260
B61	25072	4	357	4.2	230
B62	26779	15	331	17.8	235
B63	27000	9	327	10.4	228
B64	30741	5	273	8.1	202
B65	16369	23	416	6	212
B66	20496	4	433	4.4	471
B76	1287	14	1754	5.9	2319
B72	680	24	3214	23	3878
B35	781	21	2612	12	2755

Figure [SEQ Figure * ARABIC]: Clinical sample correlation results between the Theranos and CLIA B12 assays.