theranos™
redefining healthcare

Total and Free Valproic Acid (aka. VA) Assay Development Report

Theranos, Inc.

TABLE OF CONTENTS

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

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

LIST OF FIGURES

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

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

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

This assay determines the total concentration of valproic acid in human plasma or serum. The total valproic acid assay has a reportable range of 3 ug/mL to 175 ug/mL and the free valproic acid assay has a reportable range of 1.5 to 43.8 ug/mL. The assay is calibrated with material traceable to USP 1708707.

The Theranos system is also capable of measuring free valproic acid simultaneously to total valproic acid, using the Millipore Centrifree device to separate free valproic acid from valproic acid non-specially bound to plasma proteins such as albumin. This method of was evaluated for free valproic acid alongside HPLC by Liu et al, 1992. Briefly, a serum or plasma sample is applied to the Centrifree column and spun at 1000 to 2000 g for 15 minutes. The flow-through is collected and transferred to the Theranos System 3.0 cartridge. The unprocessed sample is transferred to the cartridge in a separate well. The samples are processed in parallel to determine both the total and the free valproic acid levels.

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

A biotin-labeled anti-mouse antibody coated on UltraAvidin serves as the capture surface for the competitive ELISA. The sample (serum, plasma or whole blood) is diluted and mixed with a mouse anti-VA antibody, and an enzyme-labeled conjugate. The reaction mixture is incubated on the capture surface, then the surface is washed and the substrate is incubated on the surface, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

A greater amount of VA in the sample results in lower binding of the conjugated VA to the capture antibody. Thus the signal generated by the assay is inversely proportional to the concentration of VA in the sample.

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

2.1 Cross Reactivity and Interference

The assay was tested for cross reactivity and interference from commonly co-prescribed drugs and substances with structural similarity. It should be noted that phenytoin is known to compete with valproic acid for binding to plasma proteins and a high concentration of phenytoin will increase the percentage free valproic acid, but should not impact the total valproic acid measurement. No cross reactivity or interference was observed from the test substances.

Table [SEQ Table * ARABIC]: Cross Reactivity and Interference

Test Substance	Spiked Total [VA] ug/mL	Measured Total [VA] ug/mL	CV %	% Recovery
Phenobarbitol 200 ug/mL	0	OORL		
Phenobarbitol 200 ug/mL	41	34.9	4.2	85
5-Pentanoic acid metabolite 10 ug/mL	0	OORL		
5-Pentanoic acid metabolite 10 ug/mL	41	40.2	25.1	98
Diazepam 25 ug/mL	0	OORL		
Diazepam 25 ug/mL	41	42.1	7.7	102
Ethosuximide 200 ug/mL	0	OORL		
Ethosuximide 200 ug/mL	41	35.9	13.4	87
Phenytoin 200 ug/mL	0	OORL		
Phenytoin 200 ug/mL	41	43.0	14.8	105

OORL = Out of Range Low

2.2 Normal Plasma Screen

A set of 10 normal EDTA plasma samples were screened in the assay to check for nonspecific interference as no endogenous levels are expected. All the samples yielded signal well below the lower range of detection and therefore nonspecific interference was not seen in the assay.

Table [SEQ Table * ARABIC]: Normal Plasma Screen

	Total [VA], ug/mL	
Sample ID	**Mean Conc**	**CV %**
P1	OORL	
P2	OORL	
P3	OORL	
P4	OORL	
P5	OORL	
P6	OORL	
P7	OORL	
P8	OORL	
P9	OORL	
P10	OORL	

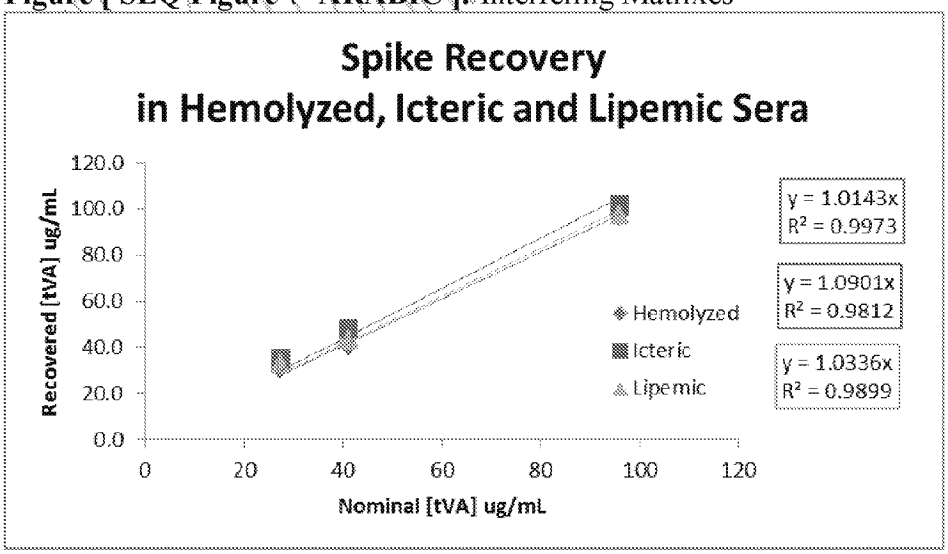
2.3 Interfering Matrixes

To determine whether icteric, Hemolyzed or lipemic samples might cause interference in the assay, these matrixes were obtain from ProMedDx and spiked with known amounts of valproic acid. Recovery was excellent in all matrixes and no interference was observed.

Table [SEQ Table * ARABIC]: Interfering Matrixes

Matrix	Spiked [tVA] ug/mL	Total [VA] Conc, ug/mL		
		Mean Conc	CV %	% Recovery
Hemolyzed	96	97.1	8.6	101
	41	40.7	9.8	99
	27	30.2	4.4	110
	0	OORL	-	-
Icteric	96	101.7	7.9	106
	41	48.2	8.0	117
	27	35.1	6.8	128
	0	OORL	-	-
Lipemic	96	97.6	15.2	102
	41	43.2	13.6	105
	27	32.9	9.0	120
	0	OORL	-	-

Figure [SEQ Figure * ARABIC]: Interfering Matrixes



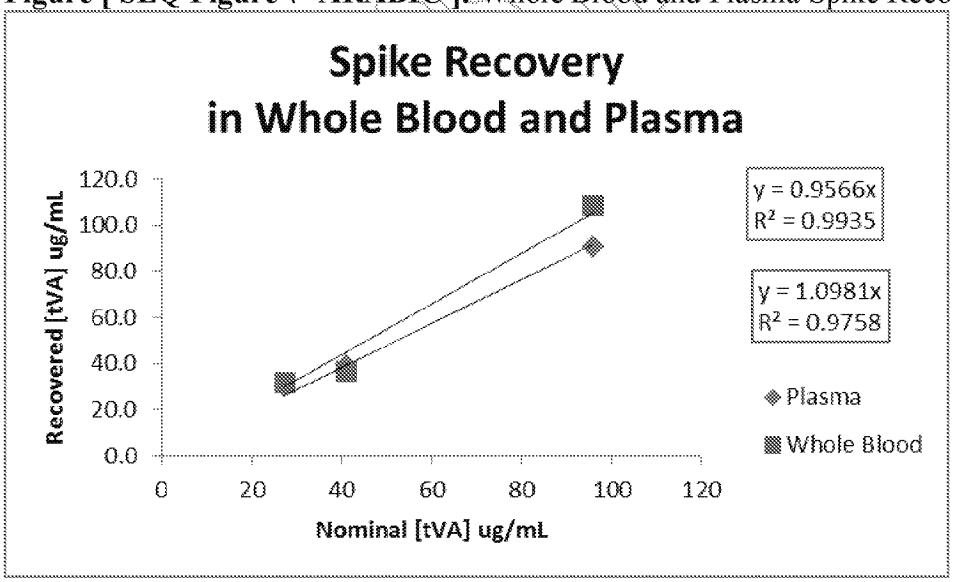
2.4 Whole Blood and Plasma Spike Recovery

Spike recovery was tested in whole blood and plasma. Spike recovery was excellent in both matrixes.

Table [SEQ Table * ARABIC]: Whole Blood and Plasma Spike Recovery

Matrix	Spiked [tVA] ug/mL	Total [VA] Conc, ug/mL		
		Mean RLU	CV %	% Recovery
Whole Blood	96	108.8	6.7	113
	41	36.4	9.3	88
	27	31.4	12.5	115
	0	OORL	-	-
Plasma	96	90.7	10.6	95
	41	39.5	8.4	96
	27	29.8	8.7	109
	0	OORL	-	-

Figure [SEQ Figure * ARABIC]: Whole Blood and Plasma Spike Recovery



2.5 Determination of LLOQ and ULOQ

LLOQ and ULOQ were determined using FDA guidelines for ELISA assay calibration. The results are shown below.

Total Valproic Acid:	Free Valproic Acid:
LLOQ = 3.0 ug/mL	LLOQ = 1.5 ug/mL
ULOQ = 175.0 ug/mL	ULOQ = 43.8 ug/mL

2.6 Clinical Correlation

The correlation of the Theranos System result under final assay conditions was compared to the reported values and the CLIA ADVIA results for the 30 clinical samples. Correlation was excellent between the Theranos result and the reported values and the ADVIA result for total valproic acid. Correlation was also excellent between the Theranos result and the ADVIA result for free valproic acid.

Table [SEQ Table * ARABIC]: Summary of Total Valproic Acid Results

	Reported, ug/mL	Theranos, ug/mL
Min	3	3.8
Max	120	93.5
Mean	69	57.4

Therapeutic Range: 50-125 µg/mL, Toxic: Greater than 150 µg/mL

	Theranos % Free VA	Theranos [Free VA] ug/mL
Min	9	< 1.5
Max	56	21.5
Mean	21	11.7

Therapeutic Range: 6 - 22 µg/mL, Toxic: Greater than 50 µg/mL

Figure [SEQ Figure * ARABIC]: Clinical Correlation Total VA Theranos to Roche Modular Reported Results

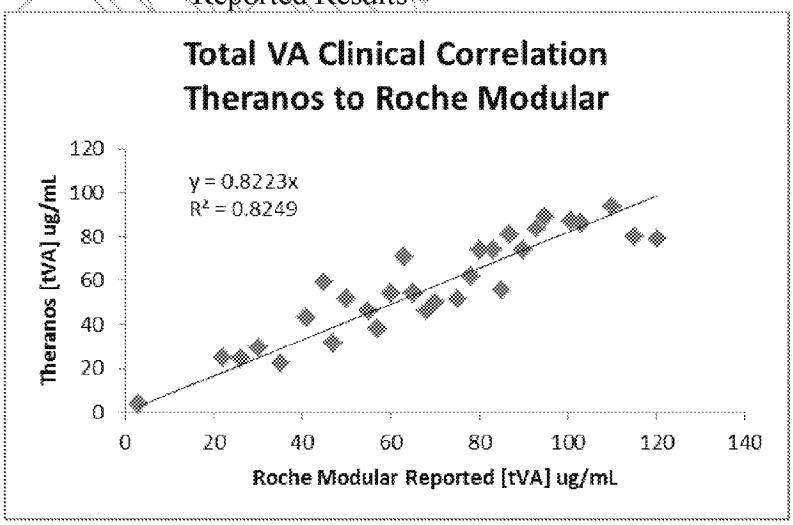


Figure [SEQ Figure * ARABIC]: Clinical Correlation Total VA Theranos to CLIA ADVIA Results

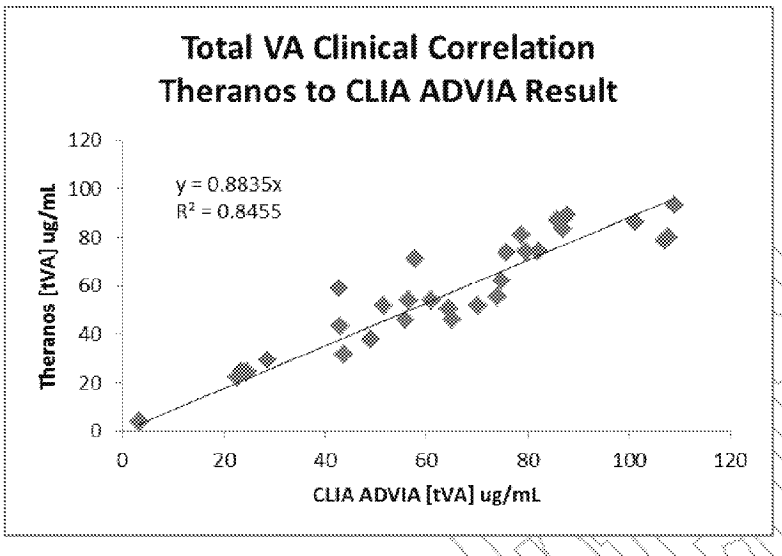


Figure [SEQ Figure * ARABIC]: Clinical Correlation Free VA Theranos to CLIA ADVIA Results

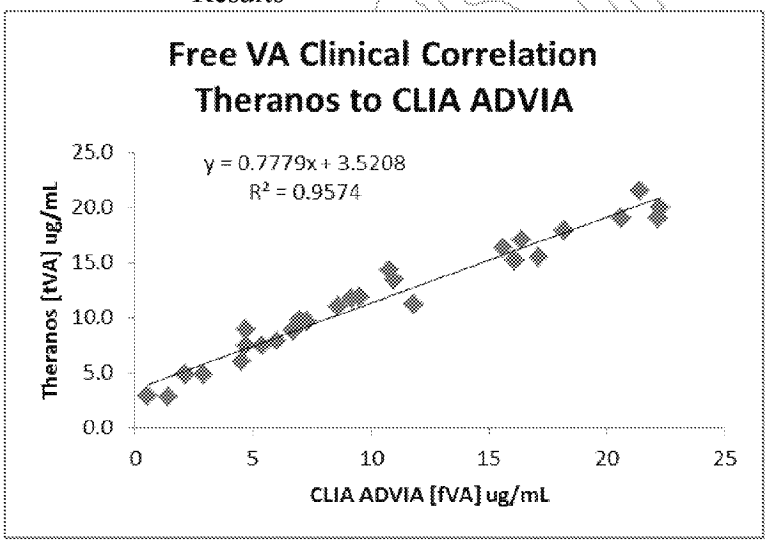


Table [SEQ Table * ARABIC]: Clinical Correlation Total Valproic Acid

Total Valproic Acid, ug/mL			
Sample ID	**Reported Roche Modula**	**CLIA ADVIA**	**Theranos**
01	35	22.7	22.4
03	30	28.7	29.6
04	78	75	61.9
05	63	57.7	71.0
06	45	42.7	59.0
07	50	51.5	51.7
08	95	87.8	89.1
09	57	49.1	38.0
10	47	43.6	31.6
11	70	64.5	50.3
12	68	65.1	46.2
13	83	75.7	73.9
14	90	82.1	74.0
15	87	78.7	81.0
16	85	74	55.5
17	75	70.1	51.9
18	65	56.5	54.0
19	22	23.5	24.8
20	80	79.6	74.0
21	55	55.9	45.9
22	41	42.9	43.1
23	93	86.9	83.7
24	3	3.3	3.8
25	60	60.9	54.1
26	26	24.5	24.5
27	110	109	93.5
28	103	101.2	86.1
29	115	107.6	80.0
30	100.6	85.8	87.0
31	120.2	107	78.8

Table [SEQ Table * ARABIC]: Clinical Correlation Free Valproic Acid

	Free Valproic Acid, ug/mL		
Sample ID	**CLIA ADVIA**	**Theranos**	**Theranos % Free**
01	2.9	4.8	21
03	1.4	2.8	9
04	17.1	15.5	25
05	NES	11.2	16
06	4.5	6.0	10
07	4.7	7.4	14
08	11	13.4	15
09	5.4	7.5	20
10	6.7	8.8	28
11	9.5	11.9	24
12	7.3	9.6	21
13	6	7.9	11
14	8.6	11.0	15
15	10.8	14.3	18
16	11.8	11.2	20
17	16.4	17.1	33
18	16.1	15.2	28
19	0.5	2.9	11
20	NES	11.8	16
21	9.2	11.7	25
22	2.1	4.9	11
23	18.2	17.9	21
24	OORL	OORL	-
25	7	9.7	18
26	4.7	8.9	36
27	21.4	21.5	23
28	15.6	16.3	19
29	20.6	19.0	24
30	22.2	19.0	22
31	22.3	20.0	25

OORL = Out of Range Low
NES = Not enough sample

2.1 Calibration Verification

To verify the calibration of the total valproic acid assay, Therapeutic Drug (TDM) controls were obtained from BioRad and Randox and tested in the Theranos system. Recovery of all controls was excellent conpared to the reported values for various predicate methods. Free valproic acid values were not provided.

Table [SEQ Table * ARABIC]: BioRad Liquicheck TDM Control Lot 25750

Level	Reported Values, ug/mL			Theranos [Total VA], ug/mL
	Roche Cobas C	Abbott Architect C	Siemens ADVIA	
1	22.7	23.5	27.8	32.3
2	74.3	79.5	77.1	90.8
3	120	120	120	99.6

Table [SEQ Table * ARABIC]: Randox TDM Calibrator Set Cat # TD3417 Lot 744TD-747TD

Level	Randox [Total VA] ug/mL	Theranos [Total VA], ug/mL
1	0.00	< 3.0
2	13.91	11.8
3	23.50	26.3
4	49.46	52.8
5	93.48	89.1
6	146.56	102.0

2.2 Effect of Anticoagulant

The Theranos System will be capable of collecting blood with both EDTA and Lithium-Heparin anticoagulant. To determine if there is any effect on the assay from the choice of anticoagulant, matched Li-Hep and EDTA samples were obtained from two donors. The resulting plasma was spiked with valproic acid and measured in the Theranos System. There was no significant different between results in EDTA or Li-Hep plasma in the total or free valproic acid assay. Either anticoagulant will be acceptable for this assay.

Table [SEQ Table * ARABIC]: Effect of Anticoagulant, Total Valproic Acid

Sample	Spiked [tVA] ug/mL	ETDA Total [VA], ug/mL			Li-Hep Total [VA], ug/mL		
		Mean Conc	CV %	% Recovery	Mean Conc	CV %	% Recovery
A	100	104.7	9.4	105	84.8	1.5	85
	50	51.3	7.7	103	31.5	3.7	63
	15	17.1	2.5	114	11.1	10.9	74
B	100	82.0	6.5	82	85.1	12.4	85
	50	43.8	1.2	88	39.3	5.0	79
	15	11.1	17.6	74	10.9	5.9	73

Table [SEQ Table * ARABIC]: Effect of Anticoagulant, Free Valproic Acid

Sample	Spiked [tVA] ug/mL	EDTA Free [VA], ug/mL		Li-Hep Free [VA], ug/mL	
		Mean Conc	CV %	Mean Conc	CV %
A	100	16.8	7.8	13.7	7.9
	50	5.8	11.9	5.2	11.6
	15	2.2	11.0	1.4	9.2
B	100	14.0	11.1	15.7	16.7
	50	4.4	12.5	5.4	4.5
	15	1.2		1.3	4.8

Figure [SEQ Figure * ARABIC]: Effect of Anticoagulant, Total Valproic Acid

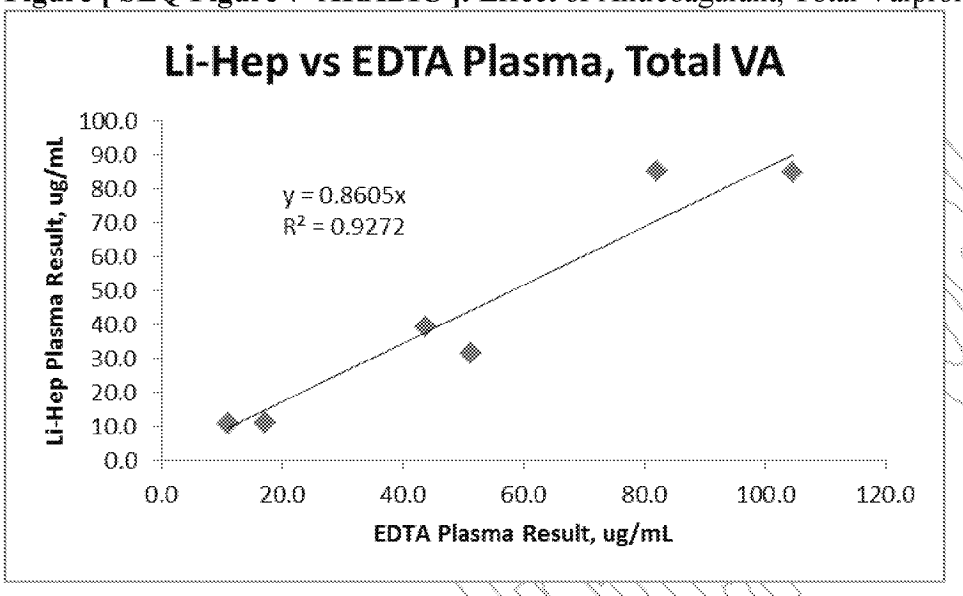
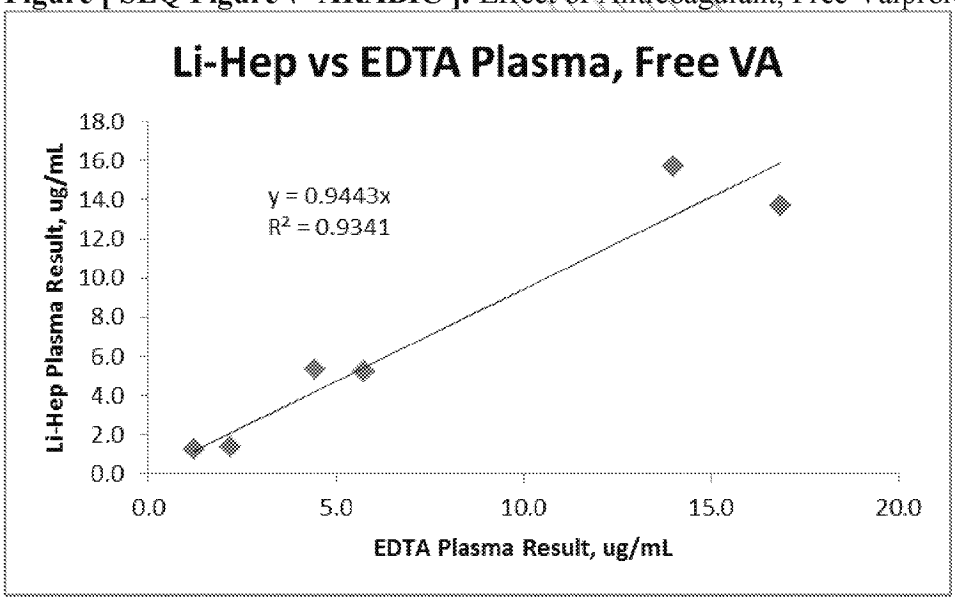


Figure [SEQ Figure * ARABIC]: Effect of Anticoagulant, Free Valproic Acid



2.3 Hematocrit Effect

To determine the hematocrit effect for the total and free valproic acid assays, whole blood was spiked with the analyte and measured in the Theranos System. Plasma was prepared from these spiked samples and then measured in the Theranos System. Total valproic acid was found to concentrate fully into plasma, resulting in approximately 1.6 fold higher results in plasma samples compared to the parent whole blood sample. Free valproic acid also concentrated into the plasma, however the hematocrit effect was lower due to the fact that whole blood and plasma are both processed into plasma water during the ultracentrifugation process. The results were consistent for both donor samples and show that a hematocrit correction factor can be used if it is desirable to compare whole blood results to plasma or serum results.

Table [SEQ Table * ARABIC]: Hematocrit Effect, Total Valproic Acid

Sample	Spiked [tVA] ug/mL	Whole Blood Total [VA], ug/mL		Plasma from WB Total [VA], ug/mL	
		Mean Conc	CV %	Mean Conc	CV %
A	40	29.7	1.4	49.5	20.4
	20	14.9	6.8	26.0	1.3
	10	10.3	6.9	13.6	24.2
B	40	36.8	12.7	62.8	5.2
	20	18.8	3.1	27.9	15.0
	10	9.4	14.1	15.2	13.5

Table [SEQ Table * ARABIC]: Hematocrit Effect, Free Valproic Acid

Sample	Spiked [tVA] ug/mL	Whole Blood Free [VA], ug/mL		Plasma from WB Free [VA], ug/mL	
		Mean Conc	CV %	Mean Conc	CV %
A	40	5.5	6.9	6.6	6.4
	20	2.2	9.2	2.7	4.9
	10	1.3	8.0	1.3	9.5
B	40	6.3	9.9	7.8	20.4
	20	2.7	8.0	2.8	20.3
	10	1.3	8.7	1.6	0.6

Figure [SEQ Figure * ARABIC]: Hematocrit Effect, Total Valproic Acid

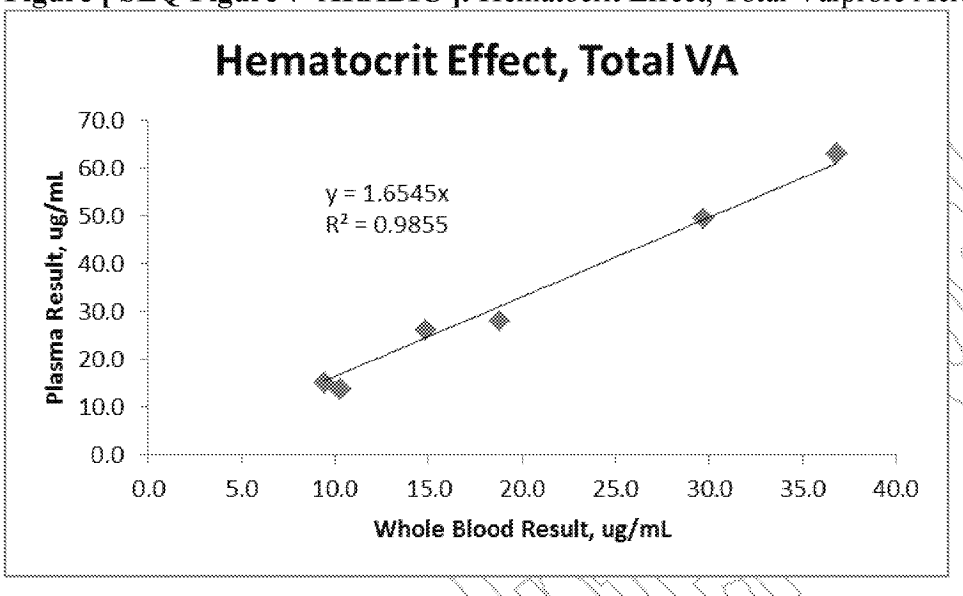
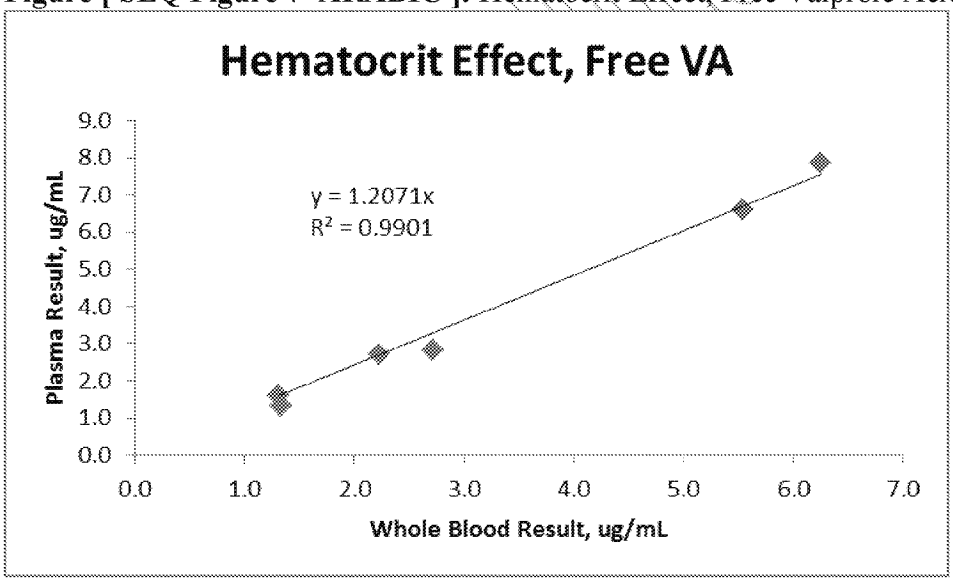


Figure [SEQ Figure * ARABIC]: Hematocrit Effect, Free Valproic Acid



2.4 HAMA and RF Interference

Samples positive for Human Anti-Mouse Antibody (HAMA) and Rheumatoid Factor (RF) were obtained from ProMedDx and tested for total and free valproic acid, to check for potential interference in these samples due to nonspecific binding to the antibody or the conjugate. No interference was observed, all samples were out of range low as expected.

Figure [SEQ Figure * ARABIC]: HAMA and RF Interference

Condition	Sample ID	Matrix	Total [VA], ug/mL	Free [VA], ug/mL
HAMA	10538678	CPD Plasma	OORL	OORL
	10538679	CPD Plasma	OORL	OORL
	10538687	Serum	OORL	OORL
	10538688	Serum	OORL	OORL
	10538690	Serum	OORL	OORL
RF	11734124	Serum	OORL	OORL
	11734125	Serum	OORL	OORL
	11734126	Serum	OORL	OORL
	11734127	Serum	OORL	OORL
	11434128	Serum	OORL	OORL

OORL = Out of Range Low

2.5 Stability

Stability studies are ongoing.

3 CONCLUSION

We have successfully developed an immunoassay for detecting total and free valproic acid in whole blood, serum and plasma.

4 REFERENCES

Liu H, Montoya JL, Forman LJ, Eggers CM, Barham CF, Delgado M. "Determination of free valproic acid: evaluation of the Centrifree system and comparison between high-performance liquid chromatography and enzyme immunoassay." Ther Drug Monit. 1992 Dec;14(6):513-21.

Hermida J, Tutor JC. "A theoretical method for normalizing total serum valproic acid concentration in hypoalbuminemic patients." J Pharmacol Sci. 2005 Apr;97(4):489-93.

Dasgupta A, Dennen D, Dean R, and McLawhon RW. "Displacement of Phenytoin from Serum Protein Carriers by Antibiotics: Studies with Ceftriaxone, Nafcillin, and Sulfamethoxazole" Clin Chem37/1, 98-100 (1991).

Perucca E, Hebdige S, Frigo G M, Gatti G, Lecchini S and Crema A. "Interaction between phenytoin and valproic acid: Plasma protein binding and metabolic effects." Clinical Pharmacology and Therapeutics (1980) 28, 779–789.

Dasgupta A. "Usefulness of monitoring free (unbound) concentrations of therapeutic drugs in patient management." Clinica Chimica Acta Volume 377, Issues 1–2, 2 February 2007, Pages 1–13.