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Prealbumin Assay Development Report

Theranos, Inc.

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

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

Prealbumin (also known as Transthyretin, TTR) is a serum protein. The functions of prealbumin include transporting thyroid hormones in plasma and cerebrospinal fluid and transporting vitamin A in plasma. It is a tetramer protein consisting four identical subunits. The molecular weight is about 52KDa with 14KDa of each subunit.

Prealbumin has a half-life in plasma of 2-3 days. It is more sensitive than serum albumin to the change of dietary intake. Therefore, prealbumin can be used as a biomarker for the nutritional evaluation and for nutritional monitoring of patients with a variety of diseases.

Normal serum concentration of prealbumin is from 15 mg/dL to 35 mg/dL. Low prealbumin level indicates acute low intake of protein. Decreased prealbumin level may also be seen in patient with severe or chronic illness, such as cancer, or liver diseases, and serious infection.

1.2 Assay specifications

This assay determines the concentration of prealbumin in human serum, plasma and whole blood. The assay has a quantification range of 15.625 µg/mL to 1000 µg/mL (1.56 mg/dL to 100 mg/dL).

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

The following assay was used as reference method:

SIEMENS ADVIA Prealbumin (PREALB)

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

A sandwich immunoassay using anti-prealbumin antibodies was developed for the quantitative determination of prealbumin in serum, plasma and whole blood.

In this assay, a goat anti-prealbumin polyclonal antibody was used as capture agent for prealbumin determination. Reaction tips were coated with Ultra-avidin first, and followed by coating of biotinlated capture antibody. Serum, plasma or whole blood samples were diluted 25000 folds with sample diluent and incubated with capture antibody coated tips. A mouse anti-prealbumin monoclonal antibody was conjugate with alkaline phosphatase and used as detection antibody. Detection antibody conjugate was incubated with reaction tips after sample incubation. After the second incubation, the tips were washed with wash buffer and incubated with AP substrate. The chemiluminescence results were measured and reported as Relative Light Units (RLU). A calibration curve was generated by plotting the measured response (RLU) vs.

concentration of each calibrator. Prealbumin concentration of unknown sample was calculated from calibration curve.

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

2.1 Verification of calibrators with reference assay

Prealbumin calibrators were analyzed by SIEMENS ADVIA prealbumin assay method to verify concentration.

Table [SEQ Table * ARABIC]: Calibrator verification by SIEMENS method

Calibrators	Nominal conc. (ug/ml)	Measured by SIEMENS (ug/ml)
1	1000	962
2	500	472
3	250	271
4	125	143
5	62.5	63
6	31.25	OORL
7	15.6	OORL
8	buffer	OORL

LLOQ of SIEMENS method: 50ug/ml

2.2 Confirmation of final assay conditions

2.2.1 Hematocrit effect and anticoagulant effect

Methods:

Whole blood, EDTA plasma and heparin plasma samples from ten donors (5 male and 5 female) were obtained in pairs from Stanford Blood Center. All samples were analyzed with final assay procedure except sample dilution being done manually. Hematocrit effect was evaluated by comparing prealbumin results in whole blood and in EDTA plasma samples from the same donor. EDTA plasma and heparin plasma from the same donor were also analyzed to compare the effect of anticoagulant.

Results:

Samples from ten donors collected in pairs of whole blood, EDA plasma and heparin plasma were analyzed. Hematocrit factor was calculated to be 1.56 from the slope of plotting prealbumin results from EDTA plasma vs. results from whole blood.

The prealbumin results from EDTA plasma and heparin plasma correlated with each other without showing significant different. This method could be used to analyze whole blood, EDTA plasma and heparin plasma.

Table [SEQ Table * ARABIC]: Prealbumin results of whole and plasma paired samples

		Whole blood			EDTA-plasma			Heparin plasma		
Sample #	Blood Center ID	Mean RLU	%CV	Calc conc (ug/ml)	Mean RLU	%CV	Calc conc (ug/ml)	Mean RLU	%CV	Calc conc (ug/ml)
M1	W070512002634	7469	20	101	13983	10	216	14933	4	233
M2	W070512002635	11983	17	180	20596	6	343	18212	16	295
M3	W070512002636	7081	20	95	14059	11	217	12003	14	180
M4	W070512002639	8669	15	122	16359	7	260	17176	14	275
M5	W070512002640	6695	12	88	13994	4	216	12294	20	186
F1	W070512002631	6875	16	91	11130	16	165	9619	14	139
F2	W070512002637	6530	29	85	9440	15	135	11670	12	174
F3	W070512002638	5844	3	74	10685	39	157	12492	16	189
F4	W070512002641	10897	14	161	15439	10	243	17023	6	273
F5	W070512002644	14615	4	228	23654	23	406	22681	16	386

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Figure [SEQ Figure * ARABIC]: Results of hematocrit effect

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Figure [SEQ Figure * ARABIC]: Results of anticoagulant effect

2.2.2 Effect of positive HAMA and RF factor

Ten HAMA positive serum samples and ten RF positive samples from PromedDx were analyzed with final assay condition. Sample dilution was done manually. All samples were also analyzed by SIEMENS method. Because the preablumin concentrations of most samples were within a narrow range, results correlation was not calculated by plotting Theranos data vs. SIEMENS data for linear regression analysis. Instead, difference between Theranos results and SIEMENS results was calculated to evaluate method compatibility with HAMA and RF positive samples.

From comparison of the difference between Theranos data and reference method, the results showed that HAMA positive and RF positive status didn't affect the prealbumin analysis severely.

Table [SEQ Table * ARABIC]: Results of analysis of HAMA positive samples and RF positive samples

	Sample	ProMedDx Lot#	Mean RLU	%CV	Theranos result (ug/ml)	SIEMENS result (ug/ml)	%Diff
HAMA positive	HAMA1	140891	22235	13	376.4	315	19.5
	HAMA2	130070	19115	9	313.1	312	0.3
	HAMA3	150139	15835	13	250.1	231	8.3
	HAMA4	130519	8408	34	117.6	139	-15.4
	HAMA5	120556	19546	4	321.6	263	22.3
	HAMA6	10580279	21576	7	362.7	257	41.1
	HAMA7	10580285	12650	7	191.9	195	-1.6
	HAMA8	10580286	10199	7	148.6	192	-22.6
	HAMA9	10580291	16216	16	257.2	219	17.5
	HAMA10	10580293	15371	1	241.5	186	29.8
RF positive	RF1	11662036	9816	15	141.9	134	5.9
	RF2	11672706	14488	5	225.2	275	-18.1
	RF3	11699268	13907	1	214.6	239	-10.2
	RF4	11715536	8133	3	112.8	154	-26.7
	RF5	11715541	17618	24	283.9	214	32.7
	RF6	11745854	13480	17	206.8	236	-12.4
	RF7	11745855	12311	27	185.9	221	-15.9
	RF8	11745857	14779	3	230.5	384	-40.0
	RF9	11745860	12261	13	185.0	164	12.8
	RF10	11745863	21782	16	367.0	296	24.0

2.2.3 Matrix effect

2.2.3.1 Hemolyzed serum samples

Six hemolyzed serum samples from Zeptometrix were analyzed. Prealbumin concentrations from Theranos method of all samples had the recovery within 80% to 120% of the results from SIEMENS method. Hemolyzed matrix didn't show matrix effect in this assay.

Table [SEQ Table * ARABIC]: Results of hemolyzed serum samples

samples	Zeptometrix Lot#	Mean RLU	%CV	Theranos result (ug/ml)	SIEMENS result (ug/ml)	%Diff
Hemo1	0107-027-00710	12823	8	195.0	181	7.7
Hemo2	0107-027-00702	12207	1	184.0	222	-17.1
Hemo3	0107-027-00707	12851	8	195.5	183	6.8
Hemo4	0107-027-00706	14652	14	228.2	201	13.5
Hemo5	0107-027-00705	12996	15	198.1	174	13.9
Hemo6	0107-027-00708	13948	17	215.3	181	19.0

2.2.3.2 Icteric samples

Five icteric serum samples from PromedDx were analyzed. Prealbumin results from Theranos method showed slightly low recovery comparing to results from SIEMENS method. The matrix effect of icteric samples was not significant.

Table [SEQ Table * ARABIC]: Results of icteric serum samples

samples	ProMedDx Lot#	Mean RLU	%CV	Theranos result (ug/ml)	SIEMENS result (ug/ml)	%Diff
Icteric1	1899979	11258	19	167.2	214	-21.9
Icteric2	1900426	15348	19	241.0	236	2.1
Icteric3	1917635	20298	21	336.7	349	-3.5
Icteric4	1920430	11480	20	171.1	248	-31.0
Icteric5	1920900	13391	12	205.2	208	-1.3

2.2.3.3 Lipemic serum samples

Six lipemic serum samples from Zeptometrix were analyzed. Prealbumin results of all samples from Theranos method were higher than SIEMENS results. These samples were also analyzed for triglycerides concentration and all of them had very high level of triglycerides. The results indicated that Theranos method might give around 2-fold higher value of prealbumin

concentration than SIEMENS method when analyzing lipemic samples with high triglycerides levels.

Table [SEQ Table * ARABIC]: Results of lipemic serum samples

samples	Zeptometrix Lot#	Mean RLU	%CV	Theranos result (ug/ml)	SIEMENS result (ug/ml)	%Diff	Triglycerides conc. (mg/dL) by CLIA lab
Lip1	0107-027-00683	19252	11	315.8	187	68.9	576
Lip2	0107-027-00684	15262	13	239.5	202	18.5	461
Lip3	0107-027-00685	14466	3	224.8	178	26.3	530
Lip4	0107-027-00687	22824	11	388.8	255	52.5	809
Lip5	0107-027-00688	23453	3	402.2	231	74.1	650
Lip6	0107-027-00689	18861	11	308.1	222	38.8	1325

2.3 Clinical sample analysis with final protocol

2.3.1 New Theranos protocol

New Theranos protocol was created and calibration curve was generated with final protocol. Data was analyzed by Theranos software.

Table [SEQ Table * ARABIC]: Calibration curve from new protocol

Sample	Conc. (ug/ml)	Mean RLU	%CV	Modulation	Cal conc (ug/ml)	%Accuracy
1	1000	96762	15	143	945	95
2	500	64745	16	96	524	105
3	250	32295	26	48	216	87
4	125	23195	5	34	146	117
5	62.5	9252	6	14	50	81
6	31.25	6216	10	9	32	101
7	15.6	3730	14	6	17	109
blank	0	674	28	1		

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Figure [SEQ Figure * ARABIC]: Calibration curve from Theranos Software analysis

Table [SEQ Table * ARABIC]: Calibration curve parameters

Model Type	4PL
Model Equation	RLU = b1+(b2-b1)/(1+(Conc/b3)^b4
Calibration Equation	conc=1937*(((286955.917)/(RLU-575.64))-1)^(1/-0.95)
b1	575.640
b2	286955.917
b3	1937.620
b4	-0.950
LLOQ	15.625 ug/ml
ULOQ	1000 ug/ml
LLOQ accuracy	109%
LLOQ precision	17.9%
ULOQ accuracy	92%
ULOQ precision	24.6%

2.3.2 Plasma recovery test with final protocol

Plasma sample P2 from PremedDx showed low prealbumin concentration so P2 was used for plasma spiking recovery test. Prealbumin standard protein was spiked into P2 at several concentration levels. All spiked samples were analyzed with final protocol. Spiking recovery was calculated by comparing nominal spiked concentration vs. measured concentration.

All five samples with prealbumin spiking levels at 500ug/ml, 400ug/ml 300ug/ml, 200ug/ml and 100ug/ml gave recovery in the range of 80% to 120% to the nominal concentration.

Table [SEQ Table * ARABIC]: Results of plasma spiking recovery

Sample #	Spiked Conc. (ug/ml)	Mean RLU	%CV	Measured conc (ug/ml)	Nominal conc (ug/ml)	%Recovery
P2		12810	15	73	73	
SP-1	500	67796	3	558	573	97
SP-2	400	53914	14	410	473	87
SP-3	300	46265	9	337	373	90
SP-4	200	33839	20	229	273	84
SP-5	100	29044	15	190	173	110

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Figure [SEQ Figure * ARABIC]: Results of plasma spiking recovery

2.3.3 Clinical sample analysis

2.3.3.1 Serum samples of ovarian cancer patients from Bioreclamation

Twenty serum samples from ovarian cancer patients were obtained from Bioreclamation. Samples were analyzed by Theranos method and SIEMENS method for prealbumin level. The result difference between Theranos method and SIEMENS method was calculated. Data from two methods correlated well. Theranos method showed slightly lower value comparing to SIEMENS results.

Table [SEQ Table * ARABIC]: Results of ovarian cancer patient serum sample analysis

Sample #	Bioreclamation Sample ID	Mean RLU	%CV	Theranos Results (ug/ml)	SIEMENS results (ug/ml)	%Difference
B1	BRH620349	42377	8	228	264	-13.7
B2	BRH620350	44430	16	242	238	1.8
B3	BRH620351	35912	15	184	209	-11.8
B4	BRH620352	34879	5	178	203	-12.4
B5	BRH620353	38936	21	204	211	-3.2
B6	BRH620354	42068	9	226	203	11.1
B7	BRH620355	34298	13	174	187	-6.9
B8	BRH620356	39669	18	209	257	-18.6
B9	BRH620357	23144	4	108	112	-3.3
B10	BRH620358	34491	22	175	206	-14.9
B11	BRH620359	30092	12	148	222	-33.2
B12	BRH620360	32448	17	163	264	-38.4
B13	BRH620361	47331	6	263	275	-4.3
B14	BRH620362	28379	21	138	201	-31.3
B15	BRH620363	13622	38	59	272	-78.5
B16	BRH620364	52225	27	301	318	-5.4

B17	BRH620365	33834	12	171	279	-38.6
B18	BRH620366	35763	13	183	216	-15.1
B19	BRH620367	34237	24	174	193	-10.0
B20	BRH620368	12783	3	54	60	-9.3

2.3.3.2 Serum and plasma samples of "low total protein" patients from PromedDx

Thirty-three serum or heparin plasma samples from "low total protein" patients were obtained from PromedDx and analyzed by both Theranos and SIEMENS methods. Data from two methods correlated well. Data reported lower than 50ug/ml from SIEMENS method were considered as "reference only" because SIEMENS method has a lower limit of quantification (LLOQ) at 50ug/ml. Percentage of difference between two methods was calculated for samples with prealbumin concentration more than 50ug/ml from SIEMENS method.

Table [SEQ Table * ARABIC]: Results of "low total protein" patient sample analysis

Sample #	ProMedDx Sample ID	Mean RLU	%CV	Theranos Results (ug/ml)	SIEMENS results (ug/ml)	%Difference
P1	11461471	8907	18	36	32 (OORL)	*
P2	11461476	15844	7	70	73	-4.5
P3	11461981	8332	9	33	10 (OORL)	*
P4	11463474	7099	5	28	19 (OORL)	*
P5	11483006	6189	12	23	7 (OORL)	*
P6	11483372	9680	13	40	25 (OORL)	*
P7	11484192	12382	16	52	37 (OORL)	*
P8	11491594	9604	6	39	24 (OORL)	*
P9	11491702	10819	8	45	46 (OORL)	*
P10	11491753	10327	13	43	35 (OORL)	*
P11	11492097	6821	12	26	8 (OORL)	*
P12	11499684	8664	12	35	14 (OORL)	*
P13	11520957	12383	20	52	43 (OORL)	*
P14	11520992	11114	7	46	32 (OORL)	*
P15	11543737	17638	4	79	60	31.5
P16	11549605	25814	5	123	116	6.3
P17	11549881	26355	12	126	115	9.9
P18	11561601	20808	2	96	86	11.2
P19	11561661	19123	27	87	81	7.0
P20	11561671	20755	6	95	115	-17.1
P21	11561712	28084	32	136	130	4.9
P22	11587939	11559	4	48	49 (OORL)	*

P23	11588890	31358	13	209	227	-8.0
P24	11588916	10039	17	41	48 (OORL)	*
P25	11589031	43850	13	238	356	-33.1
P26	11603977	10186	15	42	36 (OORL)	*
P27	11604476	14932	10	65	67	-2.9
P28	11604778	12606	12	54	43 (OORL)	*
P29	11605969	12723	15	54	46 (OORL)	*
P30	11606553	12369	26	52	52	0.8
P31	11607044	19844	23	90	91	-0.6
P32	11630114	27774	10	135	220	-38.8
P33	11630115	13813	22	59	226	-73.7

**%Difference was not calculated for sample concentration lower than 50ug/ml from SIEMENS method.*

2.3.3.3 Paired serum, EDTA plasma and Heparin plasma samples of healthy donors from Stanford Blood Center

Paired serum, EDTA plasma and heparin plasma samples were obtained from ten healthy donors (5 male and 5 female donors) at Stanford Blood Center. All samples were analyzed by both Theranos and SIEMENS methods. Data from two methods correlated well. No significant difference was observed among serum, EDTA plasma and heparin plasma samples.

Table [SEQ Table * ARABIC]: Results of Stanford healthy donor serum sample analysis

Sample #	Stanford Blood Center Sample ID	Mean RLU	%CV	Theranos Results (ug/ml)	SIEMENS results (ug/ml)	%Difference
S-M6	W070512201081	37709	23	261	322	-18.9
S-M7	W070512201082	22472	11	141	222	-36.6
S-M8	W070512201084	43406	15	311	353	-11.9
S-M9	W070512201085	32885	18	221	273	-19.0
S-M10	W070512201086	40281	15	283	294	-3.6
S-F6	W070512101024	39875	13	280	337	-17.0
S-F7	W070512101028	32855	10	221	271	-18.5
S-F8	W070512101029	39057	26	273	290	-6.0
S-F9	W070512101131	28828	7	189	235	-19.7
S-F10	W070512101134	27935	6	182	250	-27.3

Table [SEQ Table * ARABIC]: Results of Stanford healthy donor EDTA plasma sample analysis

Sample #	Stanford Blood Center Sample ID	Mean RLU	%CV	Theranos Results (ug/ml)	SIEMENS results (ug/ml)	%Difference

E-M6	W070512201081	25433	13	163	242	-32.8
E-M7	W070512201082	42427	4	302	335	-9.8
E-M8	W070512201084	40528	25	285	343	-16.8
E-M9	W070512201085	32343	4	217	261	-17.0
E-M10	W070512201086	36199	3	248	256	-3.0
E-F6	W070512101024	39096	11	273	338	-19.2
E-F7	W070512101028	31347	13	209	245	-14.8
E-F8	W070512101029	38805	14	271	272	-0.5
E-F9	W070512101131	35606	12	225	273	-17.7
E-F10	W070512101134	33320	5	243	301	-19.1

Table [SEQ Table * ARABIC]: Results of Stanford healthy donor heparin plasma sample analysis

Sample #	Stanford Blood Center Sample ID	Mean RLU	%CV	Theranos Results (ug/ml)	SIEMENS results (ug/ml)	%Difference
H-M6	W070512201081	38492	9	268	245	9.3
H-M7	W070512201082	28491	14	186	217	-14.2
H-M8	W070512201084	37225	20	257	316	-18.7
H-M9	W070512201085	32026	10	214	253	-15.4
H-M10	W070512201086	34087	4	231	264	-12.6
H-F6	W070512101024	36145	5	248	309	-19.8
H-F7	W070512101028	31203	19	208	251	-17.3
H-F8	W070512101029	29802	24	196	274	-28.3
H-F9	W070512101131	27912	26	182	225	-19.3
H-F10	W070512101134	35844	4	245	226	8.6

2.3.3.4 Summary of clinical sample analysis

Total 83 serum or plasma samples were analyzed by Theranos method final protocol. Overall, the results from Theranos method correlated with SIEMENS method reasonably.

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Figure [SEQ Figure * ARABIC]: Correlation of Theranos method vs. SIEMENS method

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Correlation of samples with 2 outliers removed

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Correlation of samples with concentration above 50ug/ml from SIEMENS method

2.4 Stability

Assay stability monitoring was done for 12 weeks.

Table [SEQ Table * ARABIC]: Results of stability monitoring

		week 0			week 2			week 4		
Calibrator	Nominal conc (ng/ml)	RLU	%CV	Back calculation	RLU	%CV	Back calculation	RLU	%CV	Back calculation
1	500	52492	17.0	489	50117	11.7	458	47479	5.5	426
2	125	17288	11.1	122	13337	6.8	90	15296	10.6	106
3	31.25	5134	10.0	29	4832	15.6	27	4632	39.2	26
4	blank	682	23.5		728	9.1		706	4.4	
					%Signal Change		**%Accuracy**	**%Signal Change**		**%Accuracy**
1	500				-4.5		91.7	-9.6		85.1
2	125				-22.9		72.3	-11.5		84.6
3	31.25				-5.9		86.2	-9.8		81.7
4	blank				6.8			3.5		

		week 8			week 12		
Calibrator	Nominal conc (ng/ml)	RLU	%CV	Back calculation	RLU	%CV	Back calculation
1	500	46528	8.3	414	43476	3.4	378
2	125	16579	16.0	116	15196	6.0	105
3	31.25	5227	23.6	30	4865	25.9	27
4	blank	812	4.7		890	45.7	
				%Accuracy			**%Accuracy**
1	500	0.0		82.8	-6.6		75.6

2	125	0.0		92.9	-8.3		84.0
3	31.25	0.0		95.3	-6.9		87.0
4	blank	0.0			9.6		

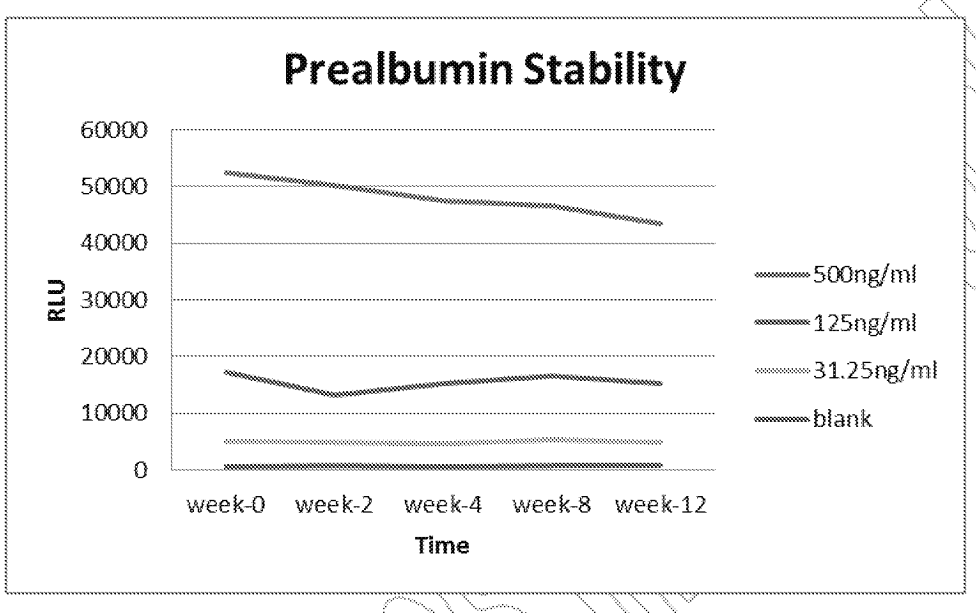

Figure [SEQ Figure * ARABIC]: Prealbumin assay stability

3 CONCLUSION

We have successfully developed an immunoassay for detecting prealbumin in serum and plasma.