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Unconjugated Estriol 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"]

Estriol (also oestriol or E3) is one of the three main estrogens. It is only produced in significant amount during pregnancy made by placenta from 16-hydroxydehydroepiandrosterone sulfate (16-OH DHEAS). Unconjugated estriol (also free estriol) can be measured in maternal blood and used as a biomarker of fetal health. If levels of unconjugated estriol are abnormally low in a pregnanct woman, this may indicate chromosomal or congenital anomalies such as Down Syndrome.

This assay determines the concentration of unconjugated estriol in plasma or serum. The assay has been calibrated to Siemens Immuelite 2000. The assay has a current reportable range of 5.57 ng/mL to 708 ng/mL.

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

The following methods have been used in house as references:

- Siemens Immulite 2000 unconjugated Estriol test provide by CLIA lab at Theranos.
- DRG, Free Estriol ELISA, Catalog # EIA-1612, certified for in vitro diagnostic use.

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

The assay is an enzyme-linked competitive immunoassay. Rabbit anti-sheep IgG biotin conjugate coated on ultra avidin serves as the capture surface. The sample is diluted and mixed with a sheep anti-estriol antibody and an alkaline phosphatase-labeled estriol conjugate (Estriol-6-AP). The reaction mixture is incubated in the liquid phase on the capture surface, unconjugated estriol in the sample competes with AP conjugated estriol (Estriol-6-AP) for a limited number of antibody binding sites. The surface is then washed and only the estriol that binds to the anti-estriol antibody is bound on the capture surface (through binding of sheep anti-estriol antibody to the anti-sheep IgG). Finally, the alkaline phosphatase substrate is incubated on the surface, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

A greater amount of unconjugated estriol in the sample results in a lower binding of the Estriol-6-AP to the caputure antibody (anti-estriol antibody). Thus the signal generated by the assay is inversely proportional to the concentration of unconjugated estriol in the sample. The higher the RLU indicates the lower level of estriol in the sample.

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Estriol (Antigen)	Fitzgerald	30-AE31
Rabbit anti-Sheep IgG (Biotin conjugate) (surface capture for Ab E19)	Fitzgerald	43C-CB1326
Goat anti-mouse IgG (Biotin conjugate) (surface capture for Ab E6)	Thermo Scientific	3185
Sheep anti-Estriol Antibody (Capture Ab E19)	USBiological	E3560-06E
Mouse anti-Estriol Antibody (Capture Ab E6)	USBiological	E3560-02E
Estriol-6-AP Conjugate	Theranos	
Estriol-3-AP Conjugate	Theranos	
Charcoal Absorbed pooled Human Serum (Steroid-Depleted Serum)	Human Biological Technologies	1016027
Phospho Glo Substrate	KPL	55-60-04
Low BSA Blocking Buffer (0.03% BSA in TBS, 0.05% Sodium Azide)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G

2 ASSAY DEVELOPMENT WITH ESTRIOL ANTIBODY E6 [TC "ASSAY OPTIMIZATION" \F C \L "2"]

2.1 Antibody and Conjugate Binding Verification on MTP

During initial assay development, an alkaline phosphatase conjugate was not available. To verify that the commercial estriol conjugates are recognized by the antibodies raised against estriol, the horseradish peroxidase (HRP) conjugates with the HRP conjugation at both 3 and 6 position of estriol were tested with 19 anti-estriol antibodies. The HRP conjugate was detected with Millipore Immobilon Chemiluminescent HRP Substrate.

A Nunc Maxisorb Microtiter plate was coated by passive absorption with 10.0, 1.0, 0.1 and 0 ug/mL of the anti-estriol antibodies and binding to a fixed concentration of the conjugates (1:1000) was tested. 16 out of the 19 tested antibodies have response to the Calbioreagents Estriol-3-HRP and Estriol-6-HRP conjugates.

Table [SEQ Table * ARABIC]: Estriol Conjugates Screened

Manufacturer	Cat #	Description
Calbioreagents	C054	Estriol-3-HRP
Calbioreagents	C154	Estriol-6-HRP

Table [SEQ Table * ARABIC]: Anti-Estriol Antibodies Screened

Theranos #	Manufacturer	Cat #	Immunogen	Clone
E1	Calbioreagents	P124	Estriol BSA conjugate	Rabbit Pab
E2	Randox	PAS9461	Estriol (6α)-BTG	Sheep PAb
E3	Randox	PAS9463	Estriol (6)-BTG	Sheep PAb
E4	US Biological	E3560-02L	Estriol BSA conjugate	10C162
E5	US Biological	E3560-02D	not reported	9F11
E6	US Biological	E3560-02E	3-CME-Estriol-BgG	9F12
E7	US Biological	E3560-02F	3-CME-Estriol-BgG	9F13
E8	US Biological	E3560-18	Estriol-3-carboxymethyl ether-BgG	8.F.106
E9	US Biological	E3560-02K	estriol-KLH	Sheep PAb
E10	Genway	GWB-E5E57E	estriol-3 conjugate	M612030
E11	Genway	GWB-863DB0	estriol-3 conjugate	M612035
E12	Genway	GWB-4257BF	estriol-3 conjugate	M612039
E13	Genway	GWB-3CA159	estriol-3 conjugate	M612034
E14	Genway	GWB-5C60B7	3-CME-Estriol-BgG	254-12333
E15	Genway	GWB-596EC8	3-CME-Estriol-BgG	254-12353
E16	Genway	GWB-300490	3-CME-Estriol-BgG	254-12343
E17	Genway	GWB-E9230D	Estriol-6 conjugate	Rabbit PAb
E18	Genway	GWB-341273	not reported	BGN/06/8601
E19	US Biological	E3560-06E	Estriol (6)-BTG	Sheep PAb

Table [SEQ Table * ARABIC]: Antibody Response to Calbioreagents Estriol HRP Conjugates (MTP)

Estriol-3-HRP Conjugate (1:1000)

Ab Conc. (ug/ml)	E 1		E 2		E 3	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	2044042	549	1256551	405	1828067	498
1	1787122	480	54148	17	75120	20
0.1	108863	29	4966	2	6966	2
0	3723		3101		3669	
	E 4		**E 5**		**E 6**	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	2529	1	3516	1	1698582	340
1	4304	1	4367	1	283398	57
0.1	4106	1	3382	1	31410	6
0	4116		3856		4993	
	E 7		**E 8**		**E 9**	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	1030507	238	1278626	311	1823863	392
1	797302	184	371255	90	802805	173
0.1	99780	23	34735	8	45982	10
0	4331		4111		4647	
	E 10		**E 11**		**E 12**	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	1175007	555	668934	364	965086	298
1	280145	132	271592	148	272716	84
0.1	16535	8	19882	11	27806	9
0	2116		1838		3240	
	E 13		**E 14**		**E 15**	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	993750	209	1132955	401	1234670	398
1	609783	128	403932	143	366736	118
0.1	88649	19	42437	15	17459	6
0	4764		2824		3099	
	E 16		**E 17**		**E 18**	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	889402	321	1890112	558	2461	1

1	427770	154	1512488	447	2884	1
0.1	57905	21	42976	13	4140	1
0	2771		3385		3399	

	E 19	
	Mean RLU	Modulation
10	1653898	52
1	182878	6
0.1	10420	0
0	31544	

Estriol-6-HRP Conjugate (1:1000)

Ab Conc. (ug/ml)	E 1		E 2		E 3	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	2393592	945	1455283	754	1863376	870
1	1903784	752	65162	34	93870	44
0.1	160384	63	5176	3	5750	3
0	2533		1929		2142	
	E 4		**E 5**		**E 6**	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	3022	1	1610	1	1518199	621
1	1437	1	1384	0	336374	138
0.1	1504	1	1933	1	34026	14
0	2180		3134		2446	
	E 7		**E 8**		**E 9**	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	764952	287	873191	305	1966786	346
1	873375	328	429207	150	1109529	195
0.1	132075	50	76059	27	44639	8
0	2666		2866		5687	
	E 10		**E 11**		**E 12**	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	908163	344	526718	276	781981	104
1	804321	305	434279	227	565772	75
0.1	69038	26	30011	16	53562	7
0	2638		1910		7514	
	E 13		**E 14**		**E 15**	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	746626	211	993287	436	800333	90

1	745862	211	521557	229	928527	105
0.1	128622	36	57220	25	61495	7
0	3539		2281		8846	

	E 16		E 17		E 18	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	669133	237	2110611	551	7063	1
1	757131	268	1675653	437	8202	1
0.1	77716	28	46922	12	9944	1
0	2825		3830		7743	

	E 19	
	Mean RLU	Modulation
10	1653092	841
1	209106	106
0.1	9468	5
0	1966	

2.2 Antibody Screen in Competitive Assay Format on Theranos System

Antibodies (at 10ng/mL) were screened for dose response to unconjugated estriol in a serum-based matrix (steroid-depleted serum) using a competitive assay format with 1:10 sample dilution and the Theranos Estriol-3-CME-AP conjugate at 1:1000,000 on the Theranos System (only Theranos Estriol-3-CME-AP available at that time, but not Theranos Estriol-6-CMO-AP). Antibodies E6, E13 and E14 gave the best modulation and were selected for further testing. These antibodies were then screened with the Bio-Rad Liquichek controls to verify response in a pure serum matrix, E6 is the antibody with all three levels of the controls in the target range, but the other two antibodies also gave reasonable response.

Table [SEQ Table * ARABIC]: Antibody Screen Using Calibrators Made in Steroid Hormone Depleted Serum

[Estriol] ng/mL	E1			E2			E3		
	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
203.6	5123	8	34	5442	6	24	994	10	53
0	175209	14		130855	17		53072	11	
	E6			E7			E8		
	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
203.6	1415	12	93	2873	15	53	2436	14	61
0	131471	14		153680	15		149442	11	
	E9			E10			E11		
	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
203.6	4875	10	32	3820	8	37	6399	7	34
0	154408	5		142960	17		217792	11	
	E12			E13			E14		
	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
203.6	4176	13	47	1512	16	95	1053	12	75
0	197277	15		143216	3		78898	13	
	E15			E16			E17		
	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
203.6	4770	18	33	5093	14	30	20549	10	13
0	157073	8		150823	10		273122	6	
	E19								
	Mean RLU	CV%	Modulation						
203.6	1430	16	30						
0	42638	3							

Table [SEQ Table * ARABIC]: Antibody Screen Using Bio-Rad Liquichek Serum Controls

Antibody E6				Back-Calculated Conc, ng/mL	
Bio-Rad Controls	**Reported Target Conc (ng/ml)**	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**
Level 1	2.34 (1.5-3.18)	38010	8	2	9
Level 2	5.96(4.41-7.51)	15224	17	5	19
Level 3	>12	5548	15	17	16

Antibody E13				Back-Calculated Conc, ng/mL	
Bio-Rad Controls	**Reported Target Conc (ng/ml)**	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**
Level 1	2.34 (1.5-3.18)	158934	9	3	18
Level 2	5.96(4.41-7.51)	91296	10	9	16
Level 3	>12	38054	8	23	7

Antibody E14				Back-Calculated Conc, ng/mL	
Bio-Rad Controls	**Reported Target Conc (ng/ml)**	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**
Level 1	2.34 (1.5-3.18)	17737	17	4	18
Level 2	5.96(4.41-7.51)	9509	16	8	19
Level 3	>12	3142	10	23	12

2.3 Training Set

Seven pregnancy serum samples from ProMedDx were tested on Theranos, Siemens Immulite 2000 at CLIA lab and DRG Free Estriol ELISA kit to evaluate the clinical correlation for antibody E6, E13 and E14 (Table 7). The results showed that antibodies E6 and E13 had good clinical correlation with DRG Free Estriol ELISA kit, but not Siemens Immulite 2000 possibly due to low test range for Siemens Immulite (<12) (Figure 1). Further, preliminary cross reactivity with two major cross reactants estradiol and estrone were tested to determine the final capture antibody. Antibody E6 was selected as final antibody for further development since Ab E13 showed cross reactivity with estradiol (Table 8).

Table [SEQ Table * ARABIC]: Clinical Sample Correlation (Training Set)

E6			Back-Calculated Conc, ng/mL			
Pregnancy Serum Sample	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**	**DRG (ng/mL)**	**Siemens (ng/mL)**
P14	4078	4	23	4	29.28	>12
S18	8125	12	11	15	15.147	8.15
P13	14723	15	5	19	11.691	10.5
P17	17958	21	4	19	9.571	10
P18	15457	21	5	20	7.733	7.45
P15	26240	8	3	9	5.06	5.61
S16	40848	8	2	10	3.09	2.2

E13			Back-Calculated Conc, ng/mL			
Pregnancy Serum Sample	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**	**DRG (ng/mL)**	**Siemens (ng/mL)**
P14	26317	11	32	8	29.28	>12
S18	43653	14	21	13	15.147	8.15
P13	76465	4	11	6	11.691	10.5
P17	95430	4	6	55	9.571	10
P18	78258	9	11	12	7.733	7.45
P15	144577	19	4	37	5.06	5.61
S16	180390	7	3	15	3.09	2.2

E14			Back-Calculated Conc, ng/mL			
Pregnancy Serum Sample	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**	**DRG (ng/mL)**	**Siemens (ng/mL)**
P14	7580	10	9	11	29.28	>12
S18	12859	10	5	10	15.147	8.15
P15	45943	13	1	14	5.06	5.61

Figure [SEQ Figure * ARABIC]: The Clinical Correlation for Theranos Training Set: Theranos vs DRG

[SHAPE * MERGEFORMAT]

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

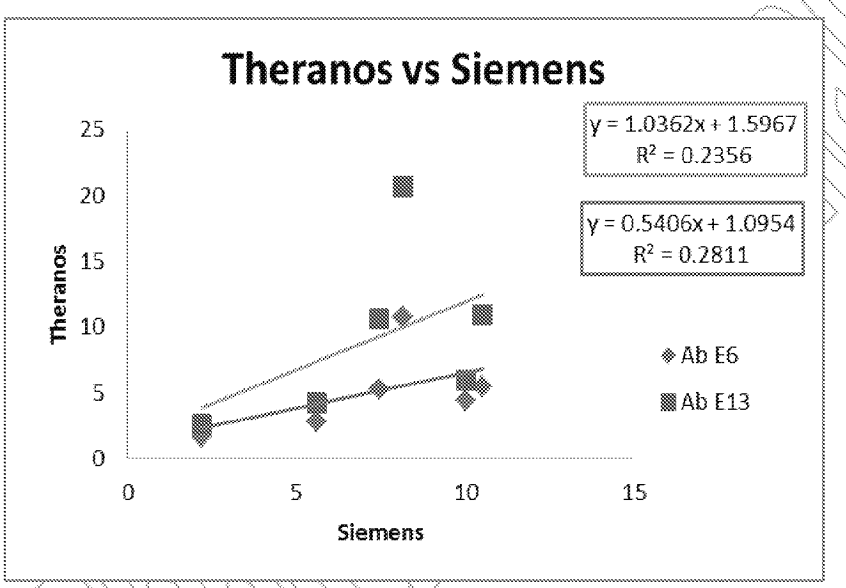


Table [SEQ Table * ARABIC]: Preliminary Cross Reactivity Test for Antibodies E6 and E13

E6				Back-Calculation, ng/mL		
[Estriol] ng/mL	Estradiol (ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
81.4	5	3540	19	100	17	122
40.7	5	6786	24	54	20	134
6.9	5	22626	9	13	13	189
0	5	71537	13	OORL		

E6				Back-Calculation, ng/mL		
[Estriol] ng/mL	Estrone (ng/mL)	Mean RLU	CV%	Mean	CV%	%Recovery

				Conc		
81.4	5	3712	15	95	16	116
40.7	5	7851	8	46	9	112
6.9	5	34196	9	7	17	99
0	5	133258	17	OORL		

E13				Back-Calculation, ng/mL		
[Estriol] ng/mL	Estradiol (ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
81.4	5	18185	16	139	22	171
40.7	5	26122	24	78	36	193
6.9	5	147427	35	5	50	75
0	5	289200	13	OORL		

2.4 AP Stabilizer

Two commercial and one Theranos alkaline phosphatase stabilizers were tested to find the best one for Theranos Estrio-3-CME-AP conjugate. In comparison with BioStab and Stabilzyme AP Stabilizers, Theranos Small Molecule AP stabilizer consisting of 5 mM Mg^{2+}, 0.1 mM Zn^{2+}, and 0.03% BSA in TBS gave better signal modulation.

Table [SEQ Table * ARABIC]: Alkaline Phosphatase Stabilizer

AP Stabilizer	[Estriol] ng/mL	Signal, RLU		
		Mean RLU	CV%	Modulation

0.03% BSA (Control)	203.6	462	23	295
	1.4	34795	15	4
	0.48	68672	21	2
	0.16	98318	15	1
	0	136146	20	
Thernaos Small Molecule AP Stabilizer	203.6	1497	17	89
	1.4	33663	6	4
	0.48	68977	17	2
	0.16	94027	8	1
	0	132674	5	
BioStab AP Stabilizer	203.6	2086	26	63
	1.4	38595	49	3
	0.48	65672	9	2
	0.16	96862	12	1
	0	131097	15	
Stabilzyme AP Stabilizer	203.6	1887	35	62
	1.4	28700	3	4
	0.48	61783	13	2
	0.16	94212	11	1
	0	116958	5	

2.5 Antibody Diluent

Three commercial antibody diluents Starting Block, Super Block, Pierce Protein-Free Blocking Buffer had been tested. Starting Block was selected as diluent based on good signal modulation.

Table [SEQ Table * ARABIC]: Antibody Diluent Test

Antibody Diluent	[Estriol] ng/mL	Signal, RLU		
		Mean RLU	CV%	Modulation
0.03% BSA	203.6	460	8	171
	81.4	533	17	148

	1.4	16467	10	5
	0	78751	7	
Starting Block	203.6	238	14	299
	81.4	531	11	134
	1.4	12111	18	6
	0	71158	5	
Super Block	203.6	284	9	219
	81.4	462	10	135
	1.4	12745	11	5
	0	62125	5	
Pierce Protein-Free Blocking Buffer	203.6	317	28	171
	81.4	510	4	106
	1.4	13521	15	4
	0	54176	16	

2.6 Capture Antibody and Theranos Estriol-3-CME-AP Conjugate Titration

Combinations of different antibody concentration and Estriol-3-CME-AP conjugation dilution were tested to select the best condition. The combination of estriol antibody E6 at 5ng/mL and Theranos Estriol-3-CME-AP at $1{:}10^6$ was selected as the best condition.

Table [SEQ Table * ARABIC]: Capture Antibody and Theranos Estriol-3AP Conjugate Titration

[CAB] ng/mL	Estrio-3-CME-AP	[Estriol] ng/mL	Mean RLU	CV%	Modulation
25	**1:10M**	203.6	151	12	115
		81.4	203	20	85

		1.4	4465	13	4
		0	17320	5	
25	**1:500K**	203.6	893	18	294
		81.4	1830	4	144
		1.4	69823	6	4
		0	262615	5	
25	**1:1M**	203.6	482	10	267
		81.4	808	15	159
		1.4	34602	10	4
		0	128773	6	
10	**1:10M**	203.6	132	5	54
		81.4	123	17	57
		1.4	1327	11	5
		0	7052	8	
10	**1:500K**	203.6	447	10	230
		81.4	813	7	126
		1.4	28620	15	4
		0	102811	5	
10	**1:1M**	203.6	320	20	200
		81.4	409	4	157
		40.7	659	15	97
		27.5	1221	5	52
		13.7	2063	10	31
		6.9	4030	17	16
		1.4	13832	9	5
		0.48	30621	17	2
		0.16	52898	20	1
		0	64044	8	
5	**1:10M**	203.6	126	7	115
		81.4	167	9	87
		1.4	2983	18	5
		0	14559	10	
5	**1:500K**	203.6	286	9	204
		81.4	429	13	136
		1.4	12790	9	5
		0	58390	27	
5	**1:1M**	203.6	181	7	224
		81.4	265	8	153
		40.7	436	7	93
		27.5	543	23	75
		13.7	1169	19	35
		6.9	2097	24	19
		1.4	8393	12	5

		0.48	14968	12	3
		0.16	28612	23	1
		0	40542	3	
1	**1:10M**	203.6	456	60	9
		81.4	234	7	18
		1.4	1172	35	4
		0	4238	10	
1	**1:500K**	203.6	1154	5	12
		81.4	1277	8	11
		1.4	3942	25	3
		0	13670	5	
1	**1:1M**	203.6	5903	9	2
		81.4	6471	10	2
		1.4	7691	14	1
		0	10181	7	

2.7 Sample Dilution and Incubation Time

The effect of sample dilution was tested with final sample dilution at 1:10 and 1:25. The sample dilution at 1:10 gave better signal modulation (Table 12). Therefore, the original 1:10 sample dilution was selected as the final sample dilution.

The effect of shorter reagent incubation time was tested with sample reaction mixture and substrate incubation time respectively at 10, 10 (original condition), 5,5 and 2, 1 minutes. With 10,10 minutes incubation, the modulation was the best (Table 13). Therefore, the Generic2_10x_coincubation protocol was selected as the final condition.

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

[Estriol] ng/mL	1:25 Sample Dilution			1:10 Sample Dilution		
	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
203.6	883	18	33	184	9	193
81.4	898	21	33	303	24	117
40.7	1188	12	25	403	10	88
27.5	1536	7	19	758	9	47
13.7	3062	8	10	1185	17	30
6.9	4205	16	7	2023	6	18
2.7	7884	6	4	4376	12	8
1.4	10605	20	3	6862	3	5
0	29197	7		35550	14	

Table [SEQ Table * ARABIC]: Effect of Incubation Time

[Estriol] ng/mL	Incubation Time 10_10			Incubation Time 5_5			Incubation Time 2_1		
	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
203.6	181	7	224	113	11	75	99	6	16
81.4	265	8	153	119	10	71	68	8	23
40.7	436	7	93	175	12	48	67	24	23
27.5	543	23	75	247	8	34	96	16	16
13.7	1169	19	35	345	15	24	110	6	14
6.9	2097	24	19	638	13	13	162	29	10
1.4	8393	12	5	1891	6	4	309	14	5
0	40542	3		8436	10		1559	16	

2.8 Clinical Samples

25 pregnancy clinical serum samples from PromedDx were tested on Theranos 3.0 System using the final protocol Generic2_10x_coincubation with antibody E6 concentration at 5ng/mL in Starting Block and Theranos Estriol-3-CME-AP dilution at $1:10^6$ in Theranos AP Stabilizer. These clinical samples were also tested with Siemens Immulite 2000 at CLIA Lab (Sample above 12 ng/mL were prediluted in PBS for the measurement on Siemens) and DRG Free Estriol ELISA kit to evaluate the clinical correlation.

Table [SEQ Table * ARABIC]: Clinical Serum Samples

Sample#	Theranos (ng/mL)	Siemens(ng/mL)	DRG kit (ng/mL)	Pregnancy week

1	11.84	12	4.122	29
2	21.14	22.5*	8.107	35
3	12.89	6.27	7.104	33
4	13.71	10.4	10.096	36
5	32.17	32.68*	29.191	36
6	10.18	5.86	5.579	28
7	9.12	8.15	7.105	32
8	15.90	17.73*	12.588	36
9	10.86	8.08	7.753	31
10	15.20	9.45	11.109	34
11	4.50	2.95	3.059	28
12	10.12	6.07	6.736	35
13	12.31	4.5	4.47	35
14	14.00	7.98	8.064	31
15	17.90	19.8*	16.713	36
16	11.41	5.35	6.842	32
17	15.98	7.68	8.214	35
18	13.24	5.15	5.314	30
19	22.14	7.82	8.386	34
20	19.94	9.6	7.75	30
21	7.57	3.19	4.288	29
22	9.74	4.72	5.91	32
23	13.87	7.2	8.273	36
24	10.71	4.66	5.184	30
25	13.60	6.11	6.213	33

*Note: Samples were prediluted in PBS before testing on Siemens Immulite 2000.

Figure [SEQ Figure * ARABIC]: Clinical Sample Correlation for Theranos and Siemens Immuelite 2000

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Clinical Sample Correlation for Theranos and DRG

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Clinical Sample Correlation for DRG and Siemens Immulite 2000

[SHAPE * MERGEFORMAT]

2.9 Cross Reactivity and Interference

Eight cross reactants had been tested on Theranos 3.0 System for cross reactivity and interference at three different levels. The results showed that estriol-3-sulfate (a conjugated estriol) had higher cross reactivity and interference with Theranos Unconjugated Estriol Assay. Therefore, the antibody E6 should be replaced by a different antibody which will have no cross reactivity problem.

				Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	Estradiol (ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
81.4	5	251	18	75	26	92
40.7	5	354	11	41	20	100
6.9	5	1418	14	6	18	83
0	5	42252	3	OORL		

				Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	Estrone (ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
81.4	5	270	10	63	14	77
40.7	5	431	33	41	85	100
6.9	5	1491	13	5	18	78
0	5	46251	7	OORL		

				Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	Estriol-3-sulfate sodium(ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
81.4	50	200	17	OORH		
40.7	50	196	11	OORH		
6.9	50	203	14	OORH		
0	50	222	17	OORH		

				Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	Estriol-16 beta D-glucuronide(ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
81.4	10	274	15	64	28	78
40.7	10	400	9	33	13	82
6.9	10	1221	12	7	18	101
0	10	4944	10	OORL		

[Estriol] ng/mL	16-Epiestriol (ng/mL)	Mean RLU	CV%	Back-Calculated Conc, ng/mL		
				Mean Conc	CV%	%Recovery
81.4	10	263	17	69	29	85
40.7	10	345	9	42	15	104
6.9	10	1187	6	7	8	104
0	10	11013	11	OORL		

[Estriol] ng/mL	Cortisol (ng/mL)	Mean RLU	CV%	Back-Calculated Conc, ng/mL		
				Mean Conc	CV%	%Recovery
81.4	50	244	13	77	25	94
40.7	50	386	16	36	23	89
6.9	50	1463	6	5	8	78
0	50	16584	12	OORL		

[Estriol] ng/mL	Progesterone (ng/mL)	Mean RLU	CV%	Back-Calculated Conc, ng/mL		
				Mean Conc	CV%	%Recovery
81.4	50	273	17	64	22	78
40.7	50	447	7	28	10	69
6.9	50	1296	13	6	18	94
0	50	18530	13	OORL		

[Estriol] ng/mL	Testosterone (ng/mL)	Mean RLU	CV%	Back-Calculated Conc, ng/mL		
				Mean Conc	CV%	%Recovery
81.4	50	268	18	68	34	84
40.7	50	412	10	32	15	79
6.9	50	1375	5	6	7	85
0	50	19094	11	OORL		

3 ASSAY DEVELOPMENT WITH ESTRIOL ANTIBODY E19

Since Theranos Estriol assay using the combination of Ab E6 and Theranos Estriol-3-AP had unacceptable cross reactivity with estriol-3-sulfate. The possibility that Estriol-6-AP might be a solution to reduce the reactivity was considered, and antibody that binds to Estriol-6-AP with higher preference than Estriol-3-AP was needed. From Table 4, it was found that estriol antibody E19 is the only one that primarily binds to Estriol-6-HRP. Therefore, we tested the hypothesis using the combination of estriol antibody E19 and Theranos Estriol-6-AP conjugate.

3.1 Antibody and Conjugate Binding Verification on MTP

To verify the binding ability of the estriol antibody E19 to Theranos Estriol-6-AP conjugates, the commercial Estriol-6-HRP (used as a control) and Theranos Estriol-6-AP conjugates at different dilution were tested with antibody E19. The ALP conjugate was detected with Theranos ALP substrate, and the HRP conjugate was detected with Millipore Immobilon Chemiluminescent HRP Substrate.

A Nunc Maxisorb Microtiter plate was coated by passive absorption with 10.0, 1.0, 0.1 and 0 ug/mL of the anti-estriol antibody E19 and binding to conjugates at different concentrations was tested. The results showed that Theranos Estriol-6-AP at 1:10000 and 1:100000 dilutions had better modulation than Estriol-6-HRP.

Table [SEQ Table * ARABIC]: Verification of Antibody E19 binding to Theranos Estriol-6-AP Conjugates (MTP)

Theranos Estriol-6-AP conjugate:

1:1000

Ab E19 Conc. (ug/ml)	Mean RLU	Modulation
10	2283100	50
1	378884	8
0.1	91381	2
0	45894	

1:10000

Ab E19 Conc. (ug/ml)	Mean RLU	Modulation
10	1794479	255
1	228003	32
0.1	34014	5
0	7029	

1:100000

Ab E19 Conc. (ug/ml)	Mean RLU	Modulation
10	1330169	823
1	138350	86
0.1	18849	12

Estriol-6-HRP conjugate:

1:1000

Ab E19 Conc. (ug/ml)	Mean RLU	Modulation
10	813204	148
1	29267	5
0	7589	1
0	5510	

0	1616	

3.2 Capture Antibody E19 and Theranos Estriol-6-CMO-AP conjugate Titration

Based on the experience with antibody E6, the protocol Generic2_10x_coincubation, Starting Block and Theranos Small Molecule AP stabilizer were used to start the assay development with antibody E19. Firstly, Theranos Estriol-6-CMO-AP conjugate titration was performed with antibody E19 at 10ng/mL, and the results showed that Theranos Estriol-6-CMO-AP at 1:100,000 is the best dilution to start with (Table 16). Next, check board titration was done with different concentrations of antibody E19 and different dilutions of Estriol-6-AP conjugate, and the combination of Estriol-6-CMO-AP conjugate dilution at 1:10,000,000 with antibody E19 concentration at 25ng/ml gave the best modulation (Table 17). Finally, the concentration of antibody E19 was titrated with Estriol-6-CMO-AP dilution at1:10,000,000 (Table 18). Based on the signal modulation, the condition with antibody E19 at 50ng/mL and Theranos Estriol-6-CMO-AP dilution at 1:10,000,000 was selected as the final condition.

Table [SEQ Table * ARABIC]: Theranos Estriol-6-AP Conjugate Titration with Antibody E19

Estrio-6-AP 1:10,000

[Estriol] ng/mL	Mean RLU	CV%	Modulation
203.6	150987	44	5
0	737187	9	

Estrio-6-AP 1:100,000

[Estriol] ng/mL	Mean RLU	CV%	Modulation
203.6	15265	27	16
0	250631	4	

Estrio-6-AP 1:1000,000

[Estriol] ng/mL	Mean RLU	CV%	Modulation
203.6	6355	22	16
0	102644	4	

Table [SEQ Table * ARABIC]: Check Board Titration of Theranos Estriol-6-AP Conjugate and Antibody E19

AP 1:1000,000, CAB E19 (25ng/ml)

[Estriol] ng/mL	Mean RLU	CV%	Modulation
81.4	12428	4	7
40.7	18354	10	5
6.9	44950	6	2
0	87426	17	

AP 1:10,000,000, CAB E19 (25ng/ml)

[Estriol] ng/mL	Mean RLU	CV%	Modulation
81.4	1244	23	8
40.7	1781	12	6
6.9	4776	7	2
0	10325	9	

AP 1:1000,000, CAB E19 (12.5ng/ml)

[Estriol] ng/mL	Mean RLU	CV%	Modulation
81.4	7978	20	7
40.7	11033	6	5
6.9	24518	6	2
0	55887	14	

AP 1:10,000,000, CAB E19 (12.5ng/ml)

[Estriol] ng/mL	Mean RLU	CV%	Modulation
81.4	1003	9	6
40.7	1145	13	6
6.9	3044	11	2
0	6396	18	

AP 1:1000,000, CAB E19 (2.5ng/ml)

[Estriol] ng/mL	Mean RLU	CV%	Modulation
40.7	3861	9	3
6.9	7623	20	2
0	12601	14	

AP 1:5,000,000, CAB E19 (2.5ng/ml)

[Estriol] ng/mL	Mean RLU	CV%	Modulation
40.7	931	8	3
0	3023	23	

Table [SEQ Table * ARABIC]: Antibody Concentration Titration with Theranos Estriol-6-CMO-AP dilution at $1:10^6$

CAB E19 (25ng/ml)

[Estriol] ng/mL	Mean RLU	CV%	Modulation
40.7	1269	12	5
6.9	3890	3	2
0	6859	18	

CAB E19 (50ng/ml)

[Estriol] ng/mL	Mean RLU	CV%	Modulation
40.7	2395	10	6
6.9	6219	13	2
0	13312	12	

CAB E19 (100ng/ml)

[Estriol] ng/mL	Mean RLU	CV%	Modulation
40.7	4091	9	6
0	22655	15	

3.3 Diluent Test

0.03% low BSA buffer and two other commercial diluents Super Block and Pierce Protein-Free Blocking Buffer were tested to see whether different diluent can improve the modulation. 0.03% BSA was selected as final diluent based on better signal modulation.

Table [SEQ Table * ARABIC]: Diluent Test

Antibody Diluent	[Estriol] ng/mL	Signal, RLU Mean RLU	CV%	Modulation
Starting Block (current diluent)	40.7	2395	10	6
	6.9	6219	13	2
	0	13312	12	
0.03% Low BSA Buffer	40.7	3328	9	7
	6.9	11878	7	2
	0	23204	13	
Super Block	40.7	2532	14	6
	6.9	9746	11	2
	0	15305	16	
Pierce Protein-Free Blocking Buffer	40.7	2544	22	4
	6.9	6469	22	2
	0	11080	10	

3.4 Incubation Time and Preincubation

The effect of shorter reagent incubation time was tested with sample reaction mixture and substrate incubation time respectively at 5, 5 minutes to compare with 10,10 minutes (current one). Preincubation of samples and antibody for 10 minutes was also tested. The results showed that the current 10, 10 minute reagent incuation time was the best one.

Table [SEQ Table * ARABIC]: Incubation Time and Preincubation

Incubation Time 5_5

[Estriol] ng/mL	Mean RLU	CV%	Modulation
40.7	1370	6	6.5
6.9	3480	36	2.6
0	8932	14	

Incubation Time 5_5, Preincubation for 10 min

[Estriol] ng/mL	Mean RLU	CV%	Modulation
40.7	1408	17	4
6.9	4092	14	1
0	5753	56	

Incubation Time 10_10

[Estriol] ng/mL	Mean RLU	CV%	Modulation
40.7	3328	9	7.0
6.9	11878	7	2.0
0	23204	13	

3.5 Sample Dilution

The effect of sample dilution was tested with final sample dilution at 1:5 to compare with current sample dilution at 1:10, and the results showed that lower sample dilution did not improve the modulation.

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

Sample Dilution at 1:5

[Estriol] ng/mL	Mean RLU	CV%	Modulation
40.7	2876	12	6.8
0	19490	22	

Sample Dilution at 1:10

[Estriol] ng/mL	Mean RLU	CV%	Modulation

40.7	3328	9	7.0
6.9	11878	7	2.0
0	23204	13	

3.6 Clinical Samples

25 pregnancy clinical serum samples from PromedDx were tested on Theranos 3.0 System using the final protocol Generic2_10x_coincubation (sample dilution at 1:10, reagent incubation time at 10, 10 minutes) with the concentration of antibody E19 at 50ng/mL in 0.03% BSA and Theranos Estriol-6-AP dilution at $1:10^7$ in Theranos Samll Molecule AP Stabilizer. These clinical samples were also tested with Siemens Immulite 2000 at CLIA Lab and DRG Free Estriol ELISA kit to evaluate the clinical correlation.

Sample#	Siemens (ng/mL)	Theranos (ng/mL)	DRG (ng/mL)
1	12	20.80	4.122
2	22.5*	27.39	8.107
3	6.27	5.68	7.104
4	10.4	10.82	10.096
5	32.68*	48.55	29.191
6	5.86	5.79	5.579
7	8.15	7.62	7.105
8	17.73*	16.52	12.588
9	8.08	10.72	7.753
10	9.45	14.96	11.109
11	2.95	3.26	3.059
12	6.07	6.79	6.736
13	4.5	9.97	4.47
14	7.98	7.83	8.064
15	19.8*	16.98	16.713
16	5.35	12.27	6.842
17	7.68	14.07	8.214
18	5.15	17.36	5.314
19	7.82	16.97	8.386
20	9.6	21.51	7.75
21	3.19	4.25	4.288
22	4.72	4.22	5.91
23	7.2	7.67	8.273
24	4.66	10.71	5.184
25	6.11	14.63	6.213

Note: Samples with * were prediluted in PBS before testing on Siemens Immulite 2000

Figure [SEQ Figure * ARABIC]: Clinical Correlation with Antibody E19 and Estriol-6-CMO-AP (Theranos vs Siemens)

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Clinical Correlation with Antibody E19 and Estriol-6-CMO-AP (Theranos vs DRG)

[SHAPE * MERGEFORMAT]

3.7 Cross Reactivity and Interference

Eight cross reactants had been tested on Theranos 3.0 System for cross reactivity and interference at three different levels. The results showed that there is no cross reactivity and interference problem with Theranos Unconjugated Estriol assay using antibody E 19 and Theranos Estriol-6-CMO-AP.

				Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	Estriol-3-sulfate sodium(ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
81.4	50	1874	4	72	5	89
40.7	50	3086	12	43	15	106
0	50	32705	3	OORL		

				Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	Estradiol (ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
81.4	5	1917	14	72	15	88
40.7	5	3408	7	39	8	95
0	5	34472	7	OORL		

				Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	Estrone (ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery

81.4	5	1759	12	78	12	96
40.7	5	3419	19	40	26	98
0	5	36348	3	OORL		

				Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	Estriol-16 beta D-glucuronide(ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
81.4	10	2041	15	67	16	83
40.7	10	3297	10	40	11	99
0	10	30905	7	OORL		

				Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	16-Epiestriol (ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
81.4	10	2156	6	63	7	77
40.7	10	3180	13	42	14	103
0	10	31091	8	OORL		

				Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	Cortisol (ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
81.4	50	2021	14	68	13	83
40.7	10	3472	15	38	20	95
0	10	39227	20	OORL		

				Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	Progesterone (ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
81.4	50	1956	12	70	11	86
40.7	50	3567	19	38	22	93
0	50	37156	9	OORL		

				Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	Testosterone (ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
81.4	50	1662	17	84	18	103
40.7	50	3419	11	39	13	95
0	50	42665	9	OORL		

3.8 Matrix Screen

11 EDTA plasma samples were screened with final condition, plasma sample #4 had low RLU and high calculated concentration. Normal and interference serum samples were also screened. The calculated concentration for normal serum samples were OORL, but hemolyzed, icteric and lipemic serum samples gave unacceptable high concentration compared with steroid-depleted serum which was used as the calibrator matrix.

Table [SEQ Table * ARABIC]: EDTA Plasma Screen

			Back-Calculated Conc, ng/mL	
Samples	**Mean RLU**	**CV%**	**Mean Conc. (ng/mL)**	**CV%**
1	19690	18	OORL	
2	24204	13	OORL	
3	24631	12	OORL	
4	7481	12	27	17
5	24041	7	OORL	
6	19364	9	OORL	
7	26664	14	OORL	
8	26700	17	OORL	
9	26913	7	OORL	
10	22130	21	OORL	
11	18571	8	OORL	

Table [SEQ Table * ARABIC]: Normal Serum Screen

Samples	Mean RLU	CV%	Mean Conc. (ng/mL)
Male Serum	25310	10	OORL
Female Serum	22148	14	OORL
Pooled Serum	19172	13	OORL
Steroid-depleted serum	26680	14	OORL

Table [SEQ Table * ARABIC]: Interference Matrices Screen

			Back-Calculated Conc, ng/mL	
Samples	Mean RLU	CV%	Mean Conc	CV%
Hemolyzed Serum	1144	15	386	37
Icteric Serum	3975	7	52	7
Lipemic Serum	10425	13	15	27
Steroid-depleted serum	28515	5	OORL	26

3.9 Trouble Shooting for Matrix Effect

To solve the matrix effect problem, different types of blocking buffer and Heterophilic Blocking Reagent-1 (HBR-1) was tested. The results showed that starting block with or without HBR-1 could solve the problem although Starting Block with 400ug/mL HBR-1 was slightly better than Starting Block alone (Table 25).

Calibrations for Starting Block with or without HBR-1 were performed and more samples were tested using the respective calibration. In general, Starting Block with HBR-1 gave a better result (Table 25).

Next, appropriate HBR-1 concentration was titrated, and the final condition is Starting Block with 200ug/mL (Table 26).

Table [SEQ Table * ARABIC]: Blocking Buffer Test

	Starting Block		Starting Block+400ug/mL HBR-1	
Samples	Mean RLU	CV%	Mean RLU	CV%
Hymolized Serum	10221	7	9182	14
EDTA plasma	8508	22	9233	8
EDTA plasma	8497	17	8782	8
Depleted Serum	10755	8	9039	14

	Suremodics Protein Free		Super Block		Low Cross Buffer	
Samples	Mean RLU	CV%	Mean RLU	CV%	Mean RLU	CV%
Hymolized Serum	11614	8	9907	17	5678	11

EDTA plasma	14537	15	13944	6	8204	13
EDTA plasma	6169	10	4502	11	3019	6
Depleted Serum	16622	12	12736	11	4974	15

Table [SEQ Table * ARABIC]: Calibration and Sample Test with Starting Block (with or without HBR-1)

Calibration with Starting Block				Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	Mean RLU	CV%	Modulation	Mean Conc	CV%	%Recovery
203.6	439	17	21.1	229	34	113
81.4	1072	9	8.6	65	9	79
40.7	1428	6	6.5	46	7	114
27.5	1930	13	4.8	31	19	114
13.7	2800	22	3.3	17	42	126
6.9	4693	3	2.0	4	12	58
2.7	5194	16	1.8	3	50	117
1.4	5690	17	1.6	2	49	162
0	9238	15		0	115	

Sample Test (Starting Block)			Back-Calculated Conc, ng/mL	
Samples	Mean RLU	CV%	Mean Conc	CV%
Hymolized Serum	10221	7	0	42
Icteric Serum	7198	18	1	89
Lipemic Serum	6435	20	2	84
EDTA plasma #3	8508	22	1	114
EDTA plasma #4	8497	17	0	72
Steroid-depleted Serum	10755	8	0	47
Bio_Rad_liquicheck Level 1	5014	10	3	36
Bio_Rad_liquicheck Level 2	4138	11	6	36
Bio_Rad_liquicheck Level 3	2005	20	30	29

Calibration with Starting Block with 400ug/mL HBR -1				Back-Calculated Conc, ng/mL		
Samples	Mean RLU	CV%	Modulation	Mean Conc	CV%	%Recovery
203.6	514	11	16.7	207	15	102
81.4	947	14	9.1	84	23	103
40.7	1618	11	5.3	33	18	81
27.5	1680	3	5.1	30	6	110
13.7	2276	18	3.8	18	41	134

6.9	3656	17	2.4	7	40	97
2.7	5211	8	1.6	3	18	101
1.4	6676	19	1.3	2	53	117
0	8595	9		1	28	

Sample Test (Starting Block with 400ug/mL HBR-1)			**Back-Calculated Conc, ng/mL**	
Samples	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**
Hymolized Serum	9182	14	1	45
Icteric Serum	8194	20	1	46
Lipemic Serum	6776	14	1	37
EDTA plasma #3	9233	8	1	22
EDTA plasma #4	8782	8	1	21
Steroid-depleted Serum	9039	14	1	34
Bio_Rad_liquicheck Level 1	4382	15	4	37
Bio_Rad_liquicheck Level 2	3761	13	6	28
Bio_Rad_liquicheck Level 3	1702	14	31	28

Table [SEQ Table * ARABIC]: Titration of HBR-1 Concentration

Starting Block + 400ug/mL HBR-1

Samples	**Mean RLU**	**CV%**
Depleted Serum	10111	3
EDTA plasma #4	7734	27
Hymolized Serum	8307	8

Starting Block + 200ug/mL HBR-1

Samples	**Mean RLU**	**CV%**
Depleted Serum	9322	14
EDTA plasma #4	8563	8
Hymolized Serum	9885	10

Starting Block + 100ug/mL HBR-1

Samples	**Mean RLU**	**CV%**
Depleted Serum	10228	9
EDTA plasma #4	7956	14

3.10 Interference Matrices

To confirm the final condition Starting Block with 200ug/mL HBR-1 can solve the matrix effect problem, more interference samples were tested. In addition, five EDTA plasma samples including sample #4 which had a problem in previous test were tested. The results showed that there is no interference from all these samples.

Table [SEQ Table * ARABIC]: Interference Matrices and EDTA Plasma Samples

Samples	Mean RLU	CV%	Mean Conc.
Depleted Serum	9322	14	OORL
Hymolized Serum	9885	10	OORL
Icteric	10286	24	OORL
Lipemic	8295	14	OORL
EDTA plasma #4	8563	8	OORL
EDTA plasma #1	8458	32	OORL
EDTA plasma #2	11383	25	OORL
EDTA plasma #3	8547	12	OORL
EDTA plasma #5	8219	10	OORL

3.11 Specificity

Five RF samples and five HAMA samples were tested for estriol level on Theranos, the results showed OORL for all these samples. The RF and HAMA samples were sent to CLIA lab to test on Siemens Immulite 2000, and all the samples are below 0.07 ng/mL.

Table [SEQ Table * ARABIC]: Specificity Test with RF and HAMA samples

Samples	Mean RLU	CV%	Theranos	Siemens Result (ng/mL)
RF#3	7329	4	OORL	< 0.07
RF#8	11079	6	OORL	< 0.07
RF#9	8165	19	OORL	< 0.07
RF #6	9346	15	OORL	< 0.07
RF #7	8585	2	OORL	< 0.07
HAMA #29	9754	13	OORL	< 0.07
HAMA #30	11133	14	OORL	< 0.07
HAMA #31	11254	13	OORL	< 0.07
HAMA #32	9388	11	OORL	< 0.07
HAMA #1	8701	13	OORL	< 0.07

3.12 Determination of LLOQ and ULOQ

Since the Starting Block with 200ug/mL HBR-1 solved the problem with interference matrices and showed good specificity for unconjugated estriol test, the protocol Generic2_10x_coincubation (sample dilution at 1:10 and reagent incubation time at 10, 10 minutes) with the concentration of antibody E19 at 50ng/mL in Starting Block + 200ug/mL HBR-1 and Theranos Estriol-6-CMO-AP at $1:10^7$ dilution in Theranos Samll Molecule AP Stabilizer was determined as final assay condition. The calibration curve (calibrator batch 2, calibator batch 1 was used for all the previous tests) was made with this final condition and the calibrator batch 2 was adjusted to Siemens Immulite 2000 at CLIA Lab.

LLOQ and ULOQ were determined using FDA guidelines for ELISA assay calibration. The ULOQ was 5.57 ng/mL and the ULOQ was 708 ng/mL.

Table [SEQ Table * ARABIC]: Standard Curve

Calibrator	Signal RLU			Back-Calculated Conc, ng/mL		
[Estriol] ng/mL	Mean RLU	CV%	Modulation	Mean Conc	CV%	%Recovery
708	476	11	19.6	679	13	96
269.2	897	16	10.4	295	29	110
133.2	1592	10	5.9	110	18	82
77.7	1776	24	5.2	98	46	126
39.75	2505	8	3.7	44	20	110
12	3747	11	2.5	16	30	130
5.57	5383	8	1.7	OORL		

0	9322	14		OORL		

Measurement	Value	Units
LLOQ	5.57	ng/mL
ULOQ	708	ng/mL
LLOQ accuracy	72	%
LLOQ precision	40.1	%
Average Residuals	11	%
Error in prediction: Best case	22	%
Error in prediction: Expected	34	%

3.13 Clinical Samples

21 pregnancy clinical serum samples from PromedDx and 6 contrived samples (clinical samples with higher estriol concentraions were not commercially available) by spking estriol into steroid-depleted serum were tested on Theranos 3.0 System using the final condition as described above and calaulated using the standard curve shown above. These clinical samples were also tested with Siemens Immulite 2000 at CLIA Lab and DRG Free Estriol ELISA kit to evaluate the clinical correlation.

Table [SEQ Table * ARABIC]: Clinical Samples

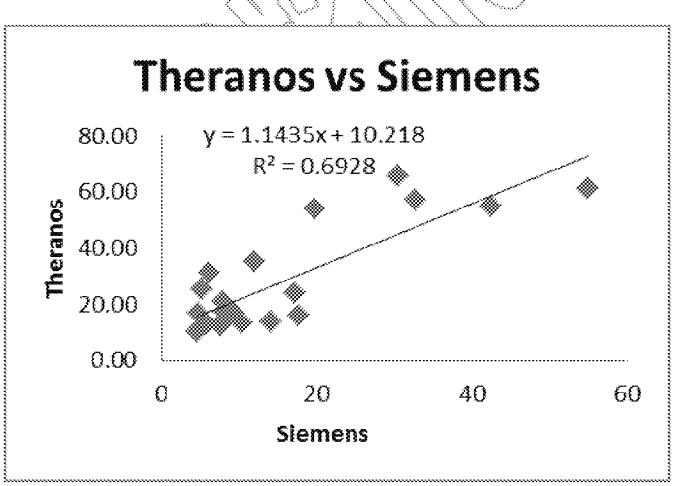
Sample#	Siemens (ng/mL)	Theranos (ng/mL)	DRG kit(ng/mL)
1	12	35.17	4.122
3	6.27	OORL	7.104
4	10.4	13.28	10.096
5	32.68*	57.21	29.191
6	5.86	12.64	5.579
7	8.15	14.23	7.105
8	17.73*	16.15	12.588
9	8.08	17.16	7.753
10	9.45	16.69	11.109
11	2.95	OORL	3.059
13	4.5	10.18	4.47

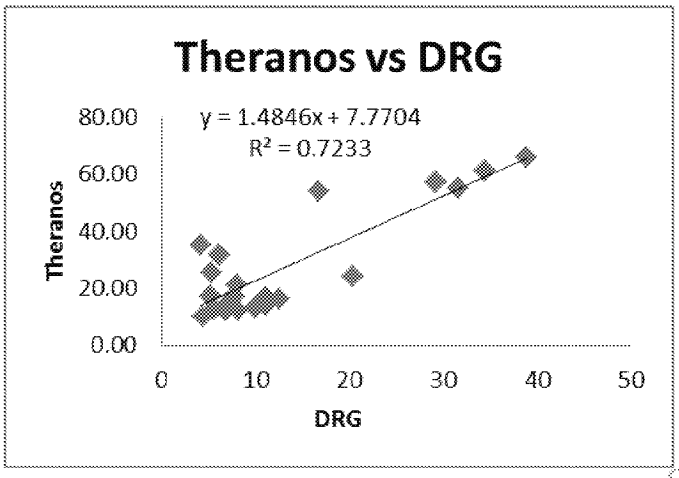
14	7.98	20.84	8.064
15	19.8*	54.06	16.713
16	5.35	12.18	6.842
17	7.68	12.34	8.214
18	5.15	25.53	5.314
21	3.19	OORL	4.288
22	4.72	OORL	5.91
23	7.2	OORL	8.273
24	4.66	17.01	5.184
25	6.11	31.41	6.213
F1	55*	61.33	34.373
F2	42.45*	55.06	31.588
FD	30.5*	65.88	38.915
FE	17.25*	24.00	20.344
FF	14.15*	13.82	11.139

Note: Samples with * were prediluted in PBS before testing on Siemens Immulite 2000

F1, F2, FD, FE, FF are contrived samples.

Figure [SEQ Figure * ARABIC]: Clinical Correlation with Antibody E19 (in Starting Block + 200ug mL HBR-1) and Estriol-6-CMO-AP (Calibrator Adjusted to Siemens Immulite 2000)





3.14 Interference Matrix Spike Recovery

Hemolyzed, icteric, and lipemic serum samples were obtained from ProMedDx. The recovery of estriol spiked into these potentially interfering matrices (1:10) was evaluated on Theranos 3.0 System. Hemolyzed, icteric and lipemic serum are not recommended for Theranos Estriol test since they tend to cause high CV although the recovery seems good on the plot for hemolyzed serum sample.

Table [SEQ Table * ARABIC]: Interference Matrix Spike Recovery

Hemolyzed serum			Back-Calculated Conc, ng/mL		
Spiked [Estriol] ng/mL in sample	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**	**%Recovery**
120	1104	29	OORH		
40	1875	8	39	15	99
20	2631	22	25	35	123
12	5143	44	10	101	85
5.5	5625	10	5	34	84
0	10552	9	OORL		

Icteric serum	Back-Calculated Conc, ng/mL

Spiked [Estriol] ng/mL in sample	Mean RLU	CV%	Mean Conc	CV%	%Recovery
120	4075	40	OORH		
40	3320	22	17	36	42
5.5	6675	19	3	43	51
0	10527	34	OORL		

Lipemic serum			Back-Calculated Conc, ng/mL		
Spiked [Estriol] ng/mL in sample	Mean RLU	CV%	Mean Conc	CV%	%Recovery
120	4803	49	OORH		
40	1892	14	40	25	100
5.5	8286	23	1	89	26
0	9139	4	OORL		

Figure [SEQ Figure * ARABIC]: Spike Recovery for Hemolyzed Serum

[SHAPE * MERGEFORMAT]

3.15 Whole Blood Screen, Spike Recovery and Hematocrit Effect

9 whole blood samples were screened on Theranos 3.0 system. Estriol concentrations for all the samples are OORL.

The whole blood recovery was determined with whole blood spiked (1:10) at five levels. The spiked whole blood samples were measured on Theranos, and the remaining spiked whole blood was centrifuged and took the plasma to test on Theranos 3.0 system to evaluate hematocrit effect. When whole blood is used for Theranos Estriol test, a new calibration curve in whole blood is needed since the whole blood samples in general gave higher RLU signal at 0 level than EDTA plasma. In addition, unconjugated estriol concentrated only slighly into plasma in comparison to whole blood – presumably as a result of its association with cells and proteins precipitated out during the preparation of the plasma.

Table [SEQ Table * ARABIC]: Whole Blood Screen

Whole Blood Samples	Mean RLU	CV%	Mean Conc
F1	18029	33	OORL
F2	14097	25	OORL
F3	13277	21	OORL
F4	15073	20	OORL
F5	19922	18	OORL
M1	19111	16	OORL
M2	15309	29	OORL
M3	11195	21	OORL
M4	14400	9	OORL

F1-F5: female, M1-M4: male

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

Whole Blood	Signal, RLU		Back-Calculated Conc, ng/mL		
Spiked [Estriol] ng/mL in sample	Mean RLU	CV%	Mean Conc	CV%	%Recovery
28	2195	18	32	25	113
20	2907	18	20	33	102
12	4421	16	9	38	77
5.5	5005	5	6	12	116
0	14615	17	OORL		

Plasma (From the whole Blood)	Signal, RLU		Back-Calculated Conc, ng/mL		
Spiked [Estriol] ng/mL in sample	Mean RLU	CV%	Mean Conc	CV%	%Recovery
28	2028	12	35	24	126
20	2649	14	23	22	116
12	3873	13	12	27	100
5.5	5647	28	6	80	105
0	9623	23	OORL		

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

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Hematocrit Effect

[SHAPE * MERGEFORMAT]

3.16 Effect of Anticoagulant

EDTA and Lithium-Heparin plasma samples were obtained from Stanford Blood Bank. The recovery of estriol spiked into plasma (1:10) was evaluated on the Theranos 3.0 System. There is

no significant difference in recovery between EDTA and Lithium-Heparin plasma. The recommended anticogulant will be EDTA plasma due to slightly better recovery compare with Lithium-Heparin plasma.

Table [SEQ Table * ARABIC]: Effect of Anticoagulant

Lithium-Heparin Plasma	Signal, RLU		Back-Calculated Conc, ng/mL		
Spiked [Estriol] ng/mL in sample	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**	**%Recovery**
40	1927	37	44	35	111
28	2684	17	23	24	82
20	3208	16	17	29	86
12	4585	10	9	18	72
0	10287	19	OORL		

EDTA Plasma	Signal, RLU		Back-Calculated Conc, ng/mL		
Spiked [Estriol] ng/mL in sample	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**	**%Recovery**
40	1859	14	41	21	103
28	2480	13	26	23	92
20	2808	18	21	27	107
12	3975	13	11	25	95
0	12094	19	OORL		

Figure [SEQ Figure * ARABIC]: Effect of Anticoagulant: Spike Recovery

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Effect of Anticoagulant: Lithium-Heparin Plasma vs EDTA Plasma

[SHAPE * MERGEFORMAT]