Prolactin
Assay Development Report

Theranos, Inc.

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Prepared by: Xiaoyan Du

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

Prolactin is secreted from the anterior pituitary gland in both men and women. Human prolactin is a peptide hormone with a molecular mass of ~22KDa. The primary functions of prolactin are to initiate breast development and to maintain lactation. During pregnancy, prolactin levels increase progressively to between 10 and 20 times of normal values. High prolactin levels can be also caused by prolactinoma, estrogens, thyrotropin-releasing hormone, and several drugs affecting dopaminergic mechanisms.

This assay is designed to detect human prolactin in human whole blood, plasma and serum. The assay has a reportable range of 2 to 500 ng/mL, and is calibrated to the WHO prolactin Third International Standard (NIBSC 84/500). Each ug of prolactin is equal to 21.2 mIU according to WHO International Standard 84/500. This is no cross-reactivity with hGH, FSH, LH and PL.

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

The following commercial ELISA kits have been used in house as predicate methods:

- Calbiotech ELISA (Cat # PR063F)
- Genway ELISA (Cat # 40-052-115021)

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

Prolactin assay is designed as a sandwich ELISA. A biotin-labeled anti-prolactin antibody is coated on an avidin surface and serves as the capture surface. The sample (whole blood, plasma or serum) is diluted and then incubated on the capture surface for 10 minutes. An alkaline phosphatase-labeled anti-prolactin antibody is incubated on the surface as detection antibody for 10 minutes. Then the surface is washed and the alkaline phosphatase substrate is incubated on the surface for 10 minutes. The resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
WHO Human Prolactin	NIBSC	84/500
Human native prolactin	Fitzgerald	30-AP05
Mouse Anti-Human Prolactin Antibody (CAb)	Fitzgerald	10-P15C
Mouse Anti-Human Prolactin Antibody (DAb)	Acris	AM09307PU-N
Alkaline Phosphatase Labeling Kit (SH)	Dojindo	LK13
Biotin Labeling Kit (SH)	Dojindo	LK10
Phospho Glo Substrate	KPL	55-60-04
Blocking Buffer (3% BSA in TBS, 0.05% Sodium Azide)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G
Pituitary hormones depleted serum	Sunny lab	SF512-2
Carbonate-bicarbonate buffer	Sigma	C3041

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

1.2 Antibody Screening (MTP)[TC "Detection Antibody Conjugate Verification" \f C \l "1"]

To determine the optimal pair for the prolactin ELISA, all combinations of 22 prolactin antibodies were tested on a microtitre plate (MTP). The screening was performed with using Fitzgerald native prolactin (Cat#30-AP05) diluted in assay buffer, 10 ug/mLof CAb and 100 ng/mL of detection antibody in blocking buffer.

Table [SEQ Table * ARABIC]: Antibody Information

Number	Vendor	Cat #	Clone #	Type
1	ARP	03-11432	n/a	Rabbit-poly
2	Novus	NBP1-36388-1.0ml	n/a	Rabbit-poly
3	Novus	NB100-62210	INN-hPRL-1 (IgG1)	Mab
4	Novus	NB100-62204	INN-hPRL-9 (IgG1)	Mab
5	Novus	NB100-73015	P1	Mab
6	AbD Serotec	7770-0369	9f3	Mab
7	Acris	AP10282PU-N	n/a	Rabbit-poly
8	Acris	AP10281PU-N	n/a	Rabbit-poly
9	AbD Serotec	0100-0665	P2	Mab
10	Acris	AM09307PU-N	PRL	Mab
11	Fitzgerald	I0-P15A	M94192	Mab
12	Fitzgerald	10-P15B	M94193	Mab
13	Fitzgerald	I0-P15C	M94194	Mab
14	Fitzgerald	70-XG80	n/a	Goat-poly
15	Fitzgerald	70-XR80	n/a	Rabbit-poly

Table [SEQ Table * ARABIC]: Summary of Antibody Screen Results

MTP	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15
D1															
D2															
D3				C4/D3		C6/D3			C9/D3	C10/D3			C13/D3		
D4									C9/D4	C10/D4		C12/D4			
D5						C6/D5				C10/D5	C11/D5	C12/D5	C13/D5		C15/D5
D6			C3/D6	C4/D6	C5/D6				C9/D6		C11/D6	C12/D6	C13/D6		C15/D6
D7															
D8															
D9										C10/D9		C12/D9	C13/D9		
D10			C3/D10	C4/D10	C5/D10	C6/D10			C9/D10		C11/D10	C12/D10	C13/D10		
D11			C3/D11	C4/D11	C5/D11	C6/D11				C10/D11		C12/D11	C13/D11		
D12			C3/D12	C4/D12	C5/D12	C6/D12			C9/D12	C10/D12	C11/D12		C13/D12		
D13			C3/D13		C5/D13	C6/D13			C9/D13	C10/D13	C11/D13	C12/D13			C15/D13
D14															
D15															

Legend:

Great responses	Fair responses	Poor responses

Table [SEQ Table * ARABIC]: Summary of Best Pairs (MTP)

Assigned Fitzgerald	C11/D6			C11/D5			C9/D4			C15/D6		
Prolactin (ng/mL)	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV
125.00	975000	1833	3	581789	993	0	543828	601	1	109559	331	1
12.50	245277	461	2	89556	153	7	68611	76	1	17091	52	5
1.25	30149	57	0	10199	17	6	11000	12	8	2074	6	3
0.00	532	1	5	586	1	21	905	1	23	331	1	4
Assigned Fitzgerald	C11/D12			C12/D13			C5/D12			C3/D6		
Prolactin (ng/mL)	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV
125.00	950153	1578	12	953534	969	4	572858	587	2	640948	289	11
12.50	256714	426	10	272684	277	12	95302	98	1	218169	98	16
1.25	12041	20	2	29433	30	2	12093	12	3	26762	12	23
0.00	602	1	18	984	1	18	976	1	18	2217	1	17
Assigned Fitzgerald	C11/D13			C3/D12			C12/D9			C6/D11		
Prolactin (ng/mL)	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV
125.00	997436	1304	4	798239	937	19	299025	586	4	702655	270	3
12.50	301000	393	5	265624	312	18	39506	77	2	136046	52	2
1.25	27330	36	4	28454	33	14	4606	9	0	18852	7	28
0.00	765	1	0	852	1	4	510	1	20	2605	1	93
Assigned Fitzgerald	C12/D6			C9/D6			C10/D12			C6/D10		
Prolactin (ng/mL)	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV
125.00	982771	1281	6	708082	921	7	891250	555	3	487207	324	14
12.50	223364	291	5	168854	220	4	179709	112	3	101724	68	16
1.25	28170	37	8	11834	15	2	22874	14	7	17759	12	6
0.00	767	1	2	769	1	39	1607	1	4	1505	1	46
Assigned Fitzgerald	C12/D11			C5/D13			C10/D5			C9/D12		
Prolactin (ng/mL)	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV
125.00	825186	1217	1	544035	902	1	873920	534	14	870820	208	2
12.50	268178	396	4	41333	69	6	289050	177	17	192334	46	1
1.25	35214	52	9	2885	5	16	33153	20	13	26528	6	2
0.00	678	1	27	603	1	6	1637	1	10	4188	1	14
Assigned Fitzgerald	C13/D6			C3/D12			C9/D10			C3/D10		
Prolactin (ng/mL)	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV
125.00	790598	1159	5	549843	806	4	266509	512	17	330983	190	16
12.50	249297	366	0	69177	101	4	36211	70	14	51282	29	11
1.25	32217	47	9	8004	12	1	4329	8	25	6854	4	23
0.00	682	1	2	656	1	10	521	1	33	1740	1	31
Assigned Fitzgerald	C3/D13			C3/D13			C10/D6			C4/D10		
Prolactin (ng/mL)	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV
125.00	948506	1177	1	**957006**	819	1	674972	439	18	226706	181	22
12.50	232319	288	3	**262424**	225	3	169745	110	15	34413	27	16
1.25	26714	33	8	**33742**	29	8	18597	12	16	4616	4	15
0.00	806	1	23	**1168**	1	23	1539	1	14	1253	1	24
Assigned Fitzgerald	C6/D13			C12/D6			C9/D5			C10/D9		
Prolactin (ng/mL)	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV
125.00	472806	1123	13	413702	739	2	601760	423	17	224778	173	16
12.50	70577	168	3	55982	100	2	123834	87	13	31633	24	13
1.25	8016	19	0	6725	12	11	12498	9	16	4286	3	22
0.00	421	1	8	560	1	1	1423	1	29	1298	1	64
Assigned Fitzgerald	C3/D11			C13/D5			C10/D3			C4/D6		
Prolactin (ng/mL)	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV
125.00	630551	1085	56	500948	739	5	214929	368	8	569257	179	18
12.50	250993	432	0	36215	53	0	38975	67	2	228321	72	18
1.25	35451	61	4	2510	4	12	4266	7	0	31758	10	32
0.00	581	1	2	678	1	10	584	1	34	3188	1	10
Assigned Fitzgerald	C4/D12			C10/D12			C13/D10			C5/D6		
Prolactin (ng/mL)	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV	Mean	Modulation	%CV
125.00	896621	1045	3	995777	663	3	397035	397	4	674186	177	15
12.50	217024	253	1	229220	153	5	47937	48	5	223504	59	19
1.25	33568	39	24	28609	19	3	6634	7	1	44597	12	12
0.00	858	1	4	1502	1	14	1092	1	10	3812	1	24

1.3 Cross Reactivity and Interference (MTP)

Prolactin is a single chain glycoprotein. Its 3-D structure is most close to hGH and placental lactogen (PL), but only shares 19.8% and 20.3% similarity in primary amino acid sequence with hGH and hPL, respectively. Despite most of the selected Ab pairs showed a negligible amount of cross reactivity, prolactin signal was largely interfered when tested in the presence of those hormones, especially with hPL and hGH. In this assay, hGH, PL, LH and FSH were chosen for both cross reactivity and interference test. Unacceptable interference was defined as greater than 120% or less than 80% of the controls. The candidate pairs with borderline or no cross reactivity/interference of the microtitre plate were chosen for the Theranos System screen.

The assay conditions were with DAb at 100 ng/mL in Blocking Buffer, CAb at 10 ug/mL. Prolactin calibrators were diluted in Blocking Buffer. The ranges of the analytes tested for cross-reactivity were as follows: hPL at 2.5 - 50 ug/mL; hGH at 2.5-50 ng/mL; hLH at 5 - 100 ng/mL; hFSH at 5 -100 ng/mL. And the doses of the substances tested for interference were hPL at 50 ug/mL; hGH at 50 ng/mL; hLH at 100 ng/mL; hFSH 5 -100 ng/mL.

Table [SEQ Table * ARABIC]: Cross Reactivity (MTP) Results

Ab pair C13/D10		Range	2.5-50 ug/mL		2.5-50 ng/mL		5-100 ng/mL		5-100 ng/mL	
Prolactin standard curve			hPL		hGH		hLH		hFSH	
Nominal ng/mL	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery
125.00	130.92	104.74	0.07	0.11	0.32	0.25	0.11	0.09	0.19	0.15
31.25	28.76	92.03	0.04	0.06	0.12	0.10	0.07	0.06	0.18	0.14
7.81	8.80	112.61	0.05	0.08	0.10	0.08	0.07	0.06	0.18	0.15
1.95	1.82	93.39	0.04	0.07	0.08	0.07	0.10	0.08	0.18	0.15
0.49	0.51	103.66	0.09	0.14	0.09	0.07	0.10	0.08	0.19	0.15
Ab pair C13/D5		Range	2.5-50 ug/mL		2.5-50 ng/mL		5-100 ng/mL		5-100 ng/mL	
Prolactin standard curve			hPL		hGH		hLH		hFSH	
Nominal ng/mL	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery
125.00	126.73	101.38	0.52	0.42	0.54	0.43	0.50	0.40	0.53	0.43
31.25	28.96	92.66	0.34	0.27	0.39	0.31	0.49	0.39	0.90	0.72
7.81	8.59	110.00	0.24	0.19	0.29	0.23	0.41	0.33	0.51	0.41
1.95	1.79	91.71	0.23	0.18	0.25	0.20	0.37	0.30	0.51	0.41
0.49	0.51	103.83	0.40	0.32	0.23	0.18	0.37	0.29	0.50	0.40
Ab pair C10/D3		Range	2.5-50 ug/mL		2.5-50 ng/mL		5-100 ng/mL		5-100 ng/mL	
Prolactin standard curve			hPL		hGH		hLH		hFSH	
Nominal ng/mL	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery
125.00	125.49	100.39	0.32	0.25	0.34	0.28	0.24	0.19	0.18	0.14
31.25	33.63	107.61	0.28	0.23	0.24	0.19	0.18	0.14	0.20	0.16
7.81	6.62	84.68	0.31	0.25	0.18	0.14	0.15	0.12	0.16	0.13
1.95	2.35	120.34	0.23	0.19	0.16	0.13	0.15	0.12	0.18	0.15
0.49	0.47	97.24	0.20	0.16	0.14	0.12	0.13	0.10	0.14	0.12
Ab pair C12/D9		Range	2.5-50 ug/mL		2.5-50 ng/mL		5-100 ng/mL		5-100 ng/mL	
Prolactin standard curve			hPL		hGH		hLH		hFSH	
Nominal ng/mL	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery
125.00	124.53	99.62	0.11	0.11	0.18	0.14	0.10	0.08	0.10	0.08
31.25	32.91	105.32	0.11	0.10	0.13	0.10	0.10	0.08	0.10	0.08
7.81	6.36	81.43	0.10	0.12	0.11	0.09	0.10	0.08	0.09	0.07
1.95	2.33	119.50	0.10	0.08	0.10	0.08	0.10	0.08	0.10	0.08
0.49	0.47	96.44	0.10	0.10	0.10	0.08	0.10	0.08	0.09	0.07

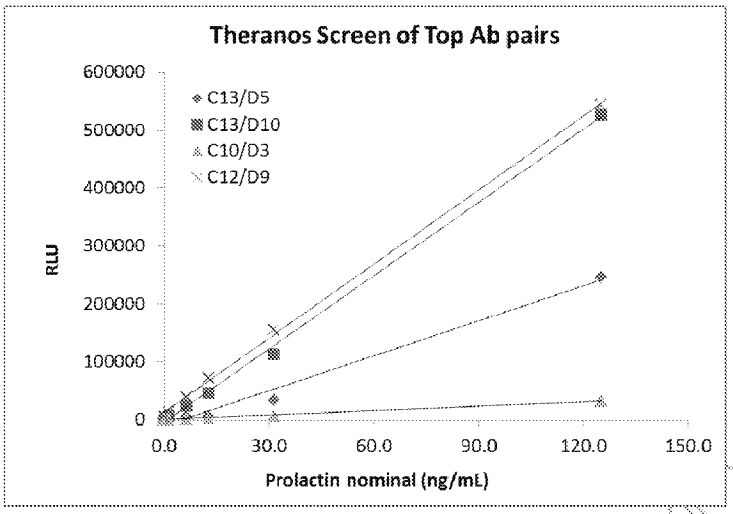
Table 6: Interference (MTP) Results

Ab pair C13/D10		Dose	50 ug/mL		50 ng/mL		100 ng/mL		300 ng/mL	
Prolactin standard curve			hPL		hGH		hLH		hFSH	
Nominal ng/mL	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery
125.00	125.45	100.36	96.69	77.35	125.04	100.03	106.02	84.82	118.41	94.73
1.25	1.25	100.18	1.18	94.00	1.70	126.36	1.13	90.18	1.25	99.83
0.00	0.27		0.81		0.43		0.86		0.31	
Ab pair C13/D5		Dose	50 ug/mL		50 ng/mL		100 ng/mL		300 ng/mL	
Prolactin standard curve			hPL		hGH		hLH		hFSH	
Nominal ng/mL	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery
125.00	125.22	100.18	93.11	74.49	114.30	91.44	103.32	82.66	109.54	87.63
1.25	1.25	100.09	1.29	103.57	1.46	117.13	1.18	94.38	1.31	104.52
0.00			0.71		0.58		0.87		0.51	
Ab pair C10/D3		Dose	50 ug/mL		50 ng/mL		100 ng/mL		300 ng/mL	
Prolactin standard curve			hPL		hGH		hLH		hFSH	
Nominal ng/mL	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery
125.00	124.89	99.91	109.03	87.22	113.08	90.46	111.55	89.24	107.54	86.03
1.25	1.25	99.97	0.86	68.73	1.17	93.56	0.79	63.06	1.20	96.33
0.00	0.39		0.36		0.47		0.49		0.61	
Ab pair C12/D9		Dose	50 ug/mL		50 ng/mL		100 ng/mL		300 ng/mL	
Prolactin standard curve			hPL		hGH		hLH		hFSH	
Nominal ng/mL	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery	Calculated	%recovery
125.00	124.78	99.82	103.95	83.16	114.13	91.30	120.10	96.08	117.45	93.96
1.25	1.25	99.91	1.20	95.60	1.23	98.67	0.98	78.56	0.97	77.36
0.00	0.19		0.50		0.37		0.54		0.23	

1.4 Theranos System Screen

The Theranos System test was done with a regular serum standard curve to evaluate the antibody pair dose responses. Prolactin calibrators (Fitzgerald) were prepared in pituitary depleted serum. The assay conditions were DAb at 100 ng/mL in Stabilzyme, CAb at 10 ug/mL in Assay buffer and a 1:10 sample dilution.

Figure [SEQ Figure * ARABIC]: Theranos system screening of top Ab pairs



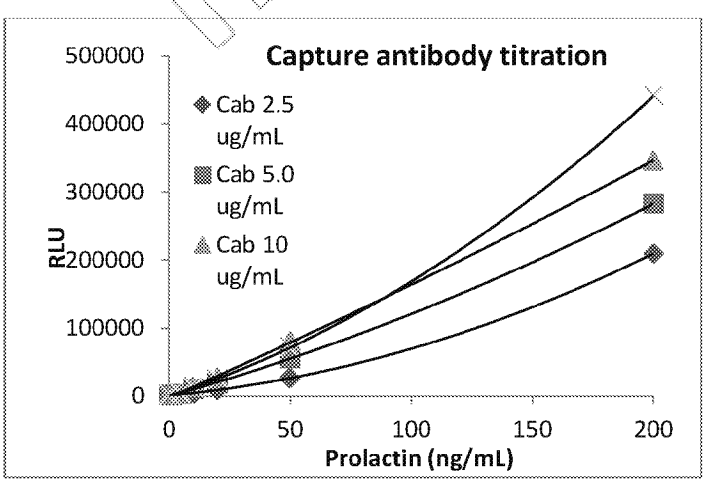
From the Theranos System screening, there were 2 pairs that both showed a good dose response and acceptable modulation in lower bottom. They are CAb 13 with DAb 10 and CAb 12 with DAb 9. In fact, Cab13/Dab10 has less interfering effect than Cab12/Dab9. As a result, Cab13/Dab10 antibody pair was chosen as the final candidate for further testing.

1.5 Capture (C13) and detection antibody (D10) titration

1.5.1 Capture Antibody Titration

The capture antibody C13 at 2.5, 5.0, 10, and 20 ug/mL were chosen for the titration. Dab 10 at 100 ng/mL in Stabilzyme buffer was used. The WHO prolactin calibrator was prepared in pituitary hormones depleted serum. Sample dilution is 1:10. Based on the results, capture antibody at 10 ug/mL yields strong dose curve responses and nice modulation at the curve bottom, therefore 10 ug/mL of Cab 13 is chosen for prolactin assay.

Figure 2: Capture antibody titration



1.5.2 Detection Antibody Titration

Next, Biostab and 3% BSA blocking were tested for detection antibody stabilizers in the Theranos system in order to know if they behave better than Stabilzyme buffer. In this test, capture antibody C13 at 10 ug/mL in 3% BSA-TBS blocking buffer was used. Sample dilution is 1:10. It turned out that Biostab displayed the best result (Figure 3). The detection antibody was titrated in Biostab buffer at 3 levels at a 1:10 sample dilution of serum calibrators in order to determine the optimal working concentration (Figure 4). The DAb concentration of 25 ng/mL provided the best modulation across the standard curve, and in particular at the lower end of the assay where sensitivity is desired. Background increased as the detection antibody was titrated higher, therefore, 25 ng/mL was chosen for detection antibody D10 as the final concentration.

Figure 3: Stabilizers optimization for detection antibody

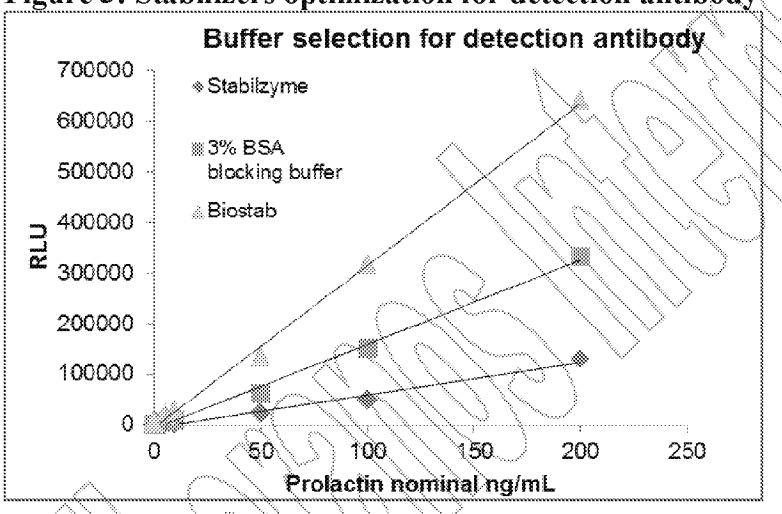
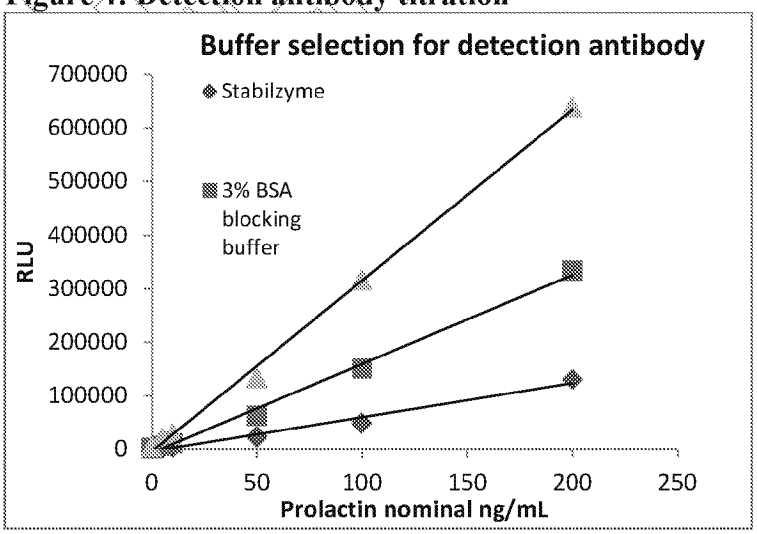


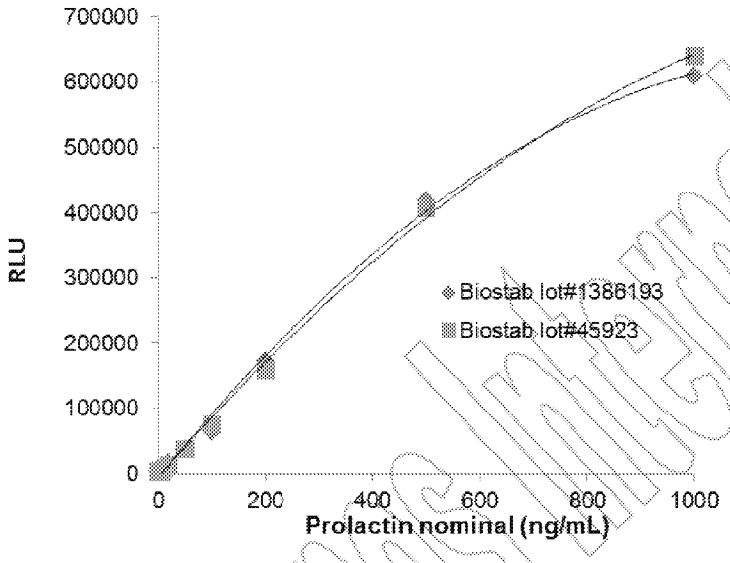
Figure 4: Detection antibody titration



1.5.3. Biostab lot-to-lot comparison

Two lots of Biostab were tested for their consistency in producing good signals. The two lots (lot#1386193 and lot#45923) tested showed very similar results.

Figure 5: Two lots of Biostab comparison



1.6 Theranos Prolactin standard curve

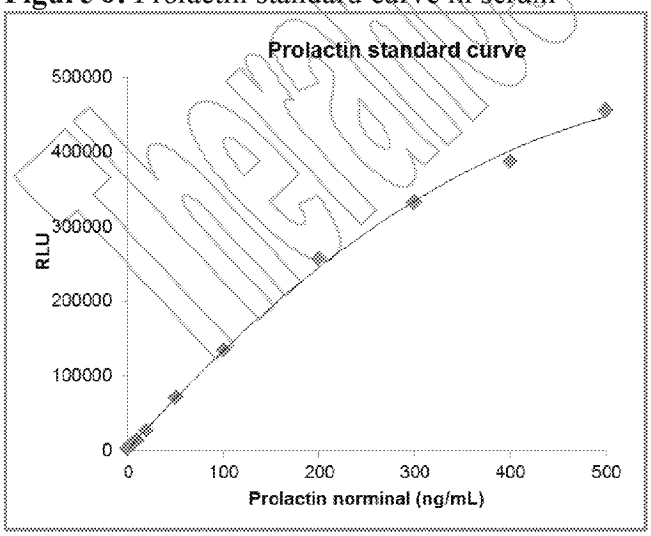
The final pair CAb 13 and Dab 10 was used for prolactin assay in the Theranos system. Using the assay conditions below, the current prolactin ELISA displays a nice detection range from 5 to 500 ng/mL.

Capture antibody (C13) at 10 ug/ml in 3% BSA blocking buffer
Detection antibody at 25 ng/ml (D10) in Biostab buffer
Calibrator from WHO prepared in depleted serum
Sample dilution 1:10

Table 7: Prolactin standard curve in serum

ng/mL	RLU1	RLU2	Ave.	CV	Modulation	Back C	Recovery
500	385245	409828	455492	11	189.6	499.9	100.0
	494442	525811					
	467573	450056					
400	426733	398136	386671	10	160.9	379.7	94.9
	372185	412408					
	394713	315854					
300	435352	395269	332716	20	138.5	299.8	99.9
	285852	282145					
	302543	295133					
200	276720	292837	257696	10	107.3	207.4	103.7
	231230	233080					
	267738	244571					
100	167865	166093	134733	20	56.1	94.5	94.5
	114234	120102					
	137476	102631					
50	90611	74875	71585	17	29.8	50.2	100.5
	79341	62176					
	60008	62497					
20	25800	24929	27697	17	11.5	20.7	103.4
	29058	30349					
	34899	21148					
10	12973	^2402	13524	14	5.6	9.5	95.1
	14691	15988					
	12742	11226					
5	9118	6621	8468	15	3.5	5.1	101.9
	9504	8630					
	^5972	^5386					
0	2637	2408	2403	9	1.0	0.4	
	2635	2443					
	2121	2173					

Figure 6: Prolactin standard curve in serum



1.7 Training set

1.7.1 Clinical samples

Twenty clinical serum samples from Bioreclamation were tested on the Theranos System with a 1:10 sample dilution. There were no pre-measured concentrations of prolactin, so the results obtained from the Theranos system were further compared to other two commercial prolactin ELISA kits (Calbiotech and Genway). The compared clinical correlations were summarized.

Prolactin level achieved by Theranos in these pregnancy samples produced acceptable correlation with two other ELISAs.

Table 8: Clinical Samples - Training Set Results

ID	P-weeks	Theranos	Genway	Calbiotech
P1	10	**30.1**	**22.4**	**51.2**
P2	27	**228.4**	**178.7**	**421.0**
P3	26	**115.5**	**97.7**	**283.8**
P4	26	**178.6**	**125.6**	**333.5**
P5	27	**181.8**	**176.0**	**426.8**
P6	29	**83.8**	**61.5**	**178.5**
P7	9	**61.6**	**41.1**	**124.8**
P8	36	**237.3**	**168.8**	**481.1**
P9	36	**257.0**	**195.6**	**480.0**
P10	24	**32.9**	**30.4**	**79.2**
P11	36	**308.7**	**180.9**	**435.6**
P12	28	**377.7**	**221.5**	**514.2**
P13	10	**84.7**	**56.4**	**170.3**
P14	26	**156.2**	**82.1**	**330.4**
P15	36	**225.2**	**139.6**	**406.5**
P16	37	**201.7**	**138.9**	**425.3**
P17	14	**73.8**	**57.5**	**153.5**
P18	36	**303.1**	**187.4**	**470.1**
P19	10	**52.1**	**40.7**	**92.6**
P20	27	**210.8**	**153.8**	**442.1**
	Mean	170.1	117.8	315.0

Conc = 10^(0.301*(LOG(RLU))^3 -4.2488*(LOG(RLU))^2 + 20.91*(LOG(RLU)) - 34.114)

Figure 7: Correlation of Theranos Result to Genway and Calbiotech ELISA Results

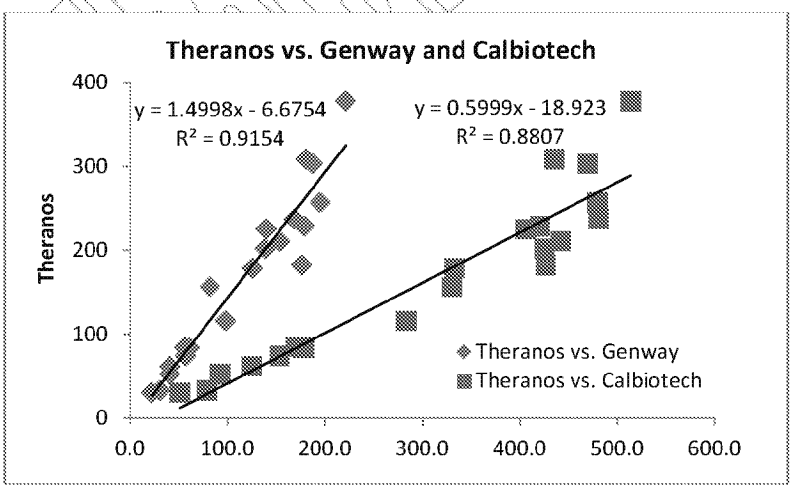
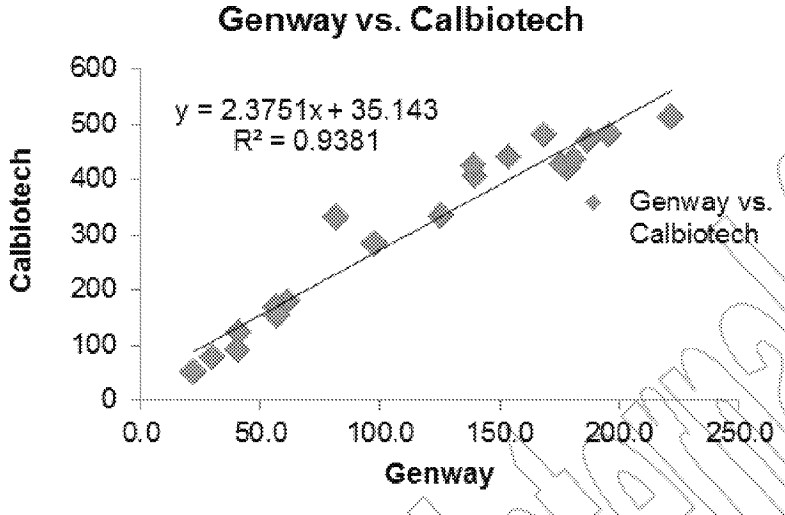


Figure 8: Correlation of Genway ELISA Result to Calbiotech Result



1.7.2 Normal EDTA-plasma

Prolactin level in ten normal subjects of EDTA-plasma was also evaluated by the Theranos system. The values were also compared to Genway and Calbiotech results. The results are well correlated among different systems.

Figure 9: Correlation of plasma samples by Theranos, Genway and Calbiotech Results

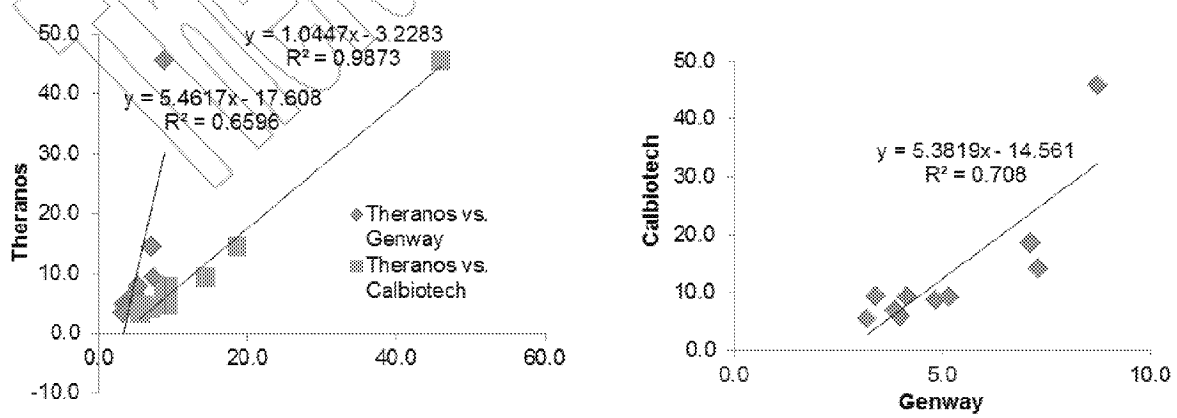


Table 9: Plasma prolactin levels tested by Theranos, Genway and Calbiotech

ng/mL	Theranos	Genway	Calbiotech
D1	5.5	4.2	9.1
D2	4.7	3.4	9.1
D3	45.7	8.7	45.9
D4	14.6	7.1	18.5
D5	6.5	4.8	8.7
D6	4.8	4.0	5.9
D7	9.4	7.3	14.2
D8	7.9	5.2	9.2
D9	3.5	3.2	5.6
D10	4.3	3.9	6.9
mean	10.7	5.2	13.3

1.7.3 Whole Blood Screen

To verify the normal range in whole blood, 10 male blood samples were screened a 1:10 sample dilution. Prolactin in the same plasma was also tested. And these results corresponded with the expected normal range in serum for male adults of 5 ng/mL.

Table10: Prolactin level in whole blood

Whole blood in EDTA		Prolactin (ng/mL)	
Summary	Blood ID	Whole blood	Plasma
1	W070511201072	**1.10**	**3.62**
2	W070511113295	**2.55**	**8.24**
3	W070511113316	**0.84**	**2.07**
4	W070511113360	**2.36**	**4.12**
5	W070511212215	**1.32**	**2.72**
6	W070511113493	**2.99**	**6.79**
7	W070511100858	**1.90**	**4.63**
8	W070511113531	**1.61**	**3.12**
9	W070511100857	**2.91**	**5.73**
10	W070511113545	**15.29**	**18.85**
Ave. prolactin level ng/mL		**3.29**	**5.99**

1.8 Effects of Sample Dilution

The effect of increasing sample dilutions was tested, with 1:10 and 1:25 dilutions. Assay conditions were DAb 100 ng/mL in Biostab and 10 ug/mL CAb. The 1:10 sample dilution provides a very linear assay with good sensitivity. At 1:25 the assay modulation is still sufficient to ensure the required sensitivity of 5 ng/mL despite of a slight lower sentitivity.

Figure 10: Effects of sample dilution: 1:10 vs. 1:25

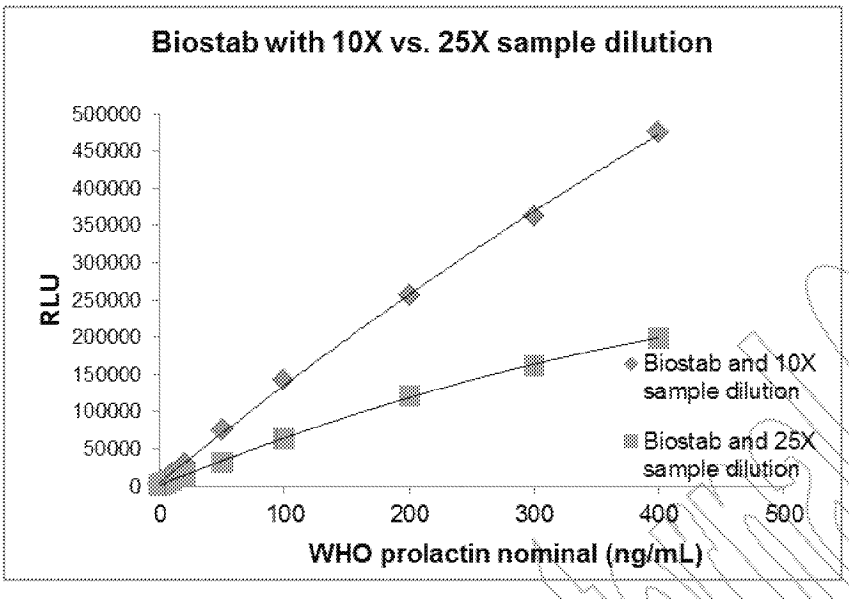


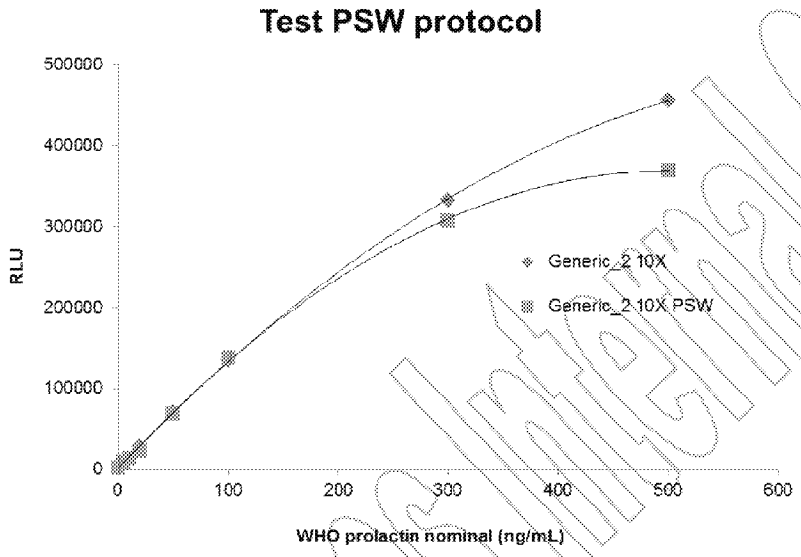
Table 11: Effect of sample dilution

Prolactin	Biostab-Dab 25 ng/mL 10X Sample dilution					Biostab-Dab 25 ng/mL 25X Sample dilution				
ng/mL	Tip1	Tip2	Ave.	CV	Modulation	Tip3	Tip4	Ave.	CV	Modulation
400	502906	482117	476136	3.3	192.7	183435	190924	199297	7.0	122.9
	475455	473056				187998	217025			
	457804	465480				213362	203038			
300	338887	375186	361771	4.2	146.4	^200844	46	162027	1.5	99.9
	349201	378101				163963	159349			
	365273	363978				162769	70680			
200	302056	306431	255905	22.5	103.6	106974	126144	121633	11.0	75.0
	^317302	336547				123262	105337			
	225296	189839				127305	140779			
100	140499	10	142130	3.5	57.5	71449	74134	62667	14.4	38.6
	144718	138533				54965	53428			
	137286	149616				65925	56103			
50	82869	82964	75815	14.6	30.7	35150	32275	32073	9.5	19.8
	66849	58401				34207	33196			
	87033	76775				30965	26649			
20	35449	28970	28893	17.2	11.7	15149	13901	14466	8.0	8.9
	23470	23363				15247	16007			
	33420	28688				13376	13119			
10	18824	17785	16193	11.0	6.6	7366	7171	7056	4.4	4.4
	15773	14022				6489	7027			
	15632	15119				7027	7255			
5	8872	8806	7799	11.3	3.2	6018	5917	5050	15.7	3.1
	7469	7624				5044	4888			
	7408	6616				4366	4064			
2	6055	4963	5379	8.8	2.2	2945	2498	2543	15.7	1.6
	5817	5454				2922	6			
	4959	5026				2352	1998			
0	2500	2474	2471	14.3	1.0	1647	1715	1622	22.0	1.0
	2060	2077				1322	1125			
	2823	2889				2136	1787			

1. 9 Effects of Post Sample Wash

Post sample wash was also tested. When sample dilution 1:10 was used, post sample wash did not generate additional benefit. Instead, it lost signal at higher doses of the standard curve.

Figure 11: Post Sample Wash Effects



1. 10 Effects of Coating Buffer

To find out the suitable buffer for coating capture antibody conjugates, Sea block, Super block and Starting block were tested. In comparison to 3% BSA blocking buffer, Sea block buffer produced comparable result but with less satisfaction from Super block and Starting block buffers.

Table 12: Coating Buffer Effects

Buffers	3% BSA blocking buffer			Sea block buffer			Super block buffer			Starting block buffer		
ng/mL	Ave. RLU	CV	Modulation	Ave. RLU	CV	Modulation	Ave. RLU	CV	Modulation	Ave. RLU	CV	Modulation
500	460374	5.0	187.7	486008	2.9	185.1	488757	8.9	197.5	518103	9.8	155.3
200	264513	8.2	107.9	282010	8.5	107.4	226684	17.0	91.6	242798	13.6	72.8
50	59995	9.3	24.5	73869	5.7	28.1	62337	11.6	25.2	69501	11.7	20.8
20	24901	17.7	10.2	30291	12.7	11.5	26455	15.0	10.7	31453	20.0	9.4
5	7450	12.6	3.0	9357	12.0	3.6	7118	10.9	2.9	8448	12.6	2.5
0	2452	13.3	1.0	2625	14.0	1.0	2474	23.8	1.0	3335	4.2	1.0

1. 11 Effects of Reagent Incubation Time

The effect of shorter reagent incubation times was tested with 10-10-10 minute and 5-5-5 minute of sample, detection conjugate, and substrate incubations respectively. Assay conditions were DAb 25g/mL in Biostab, 10 ug/mL CAb, and 1:10 sample dilution. Shortening the incubation times to 5 minutes decreased the modulation across the standards. Therefore, a 10-10-10 assay format is optimal.

Figure 12: Effects of Reagents Incubation Time

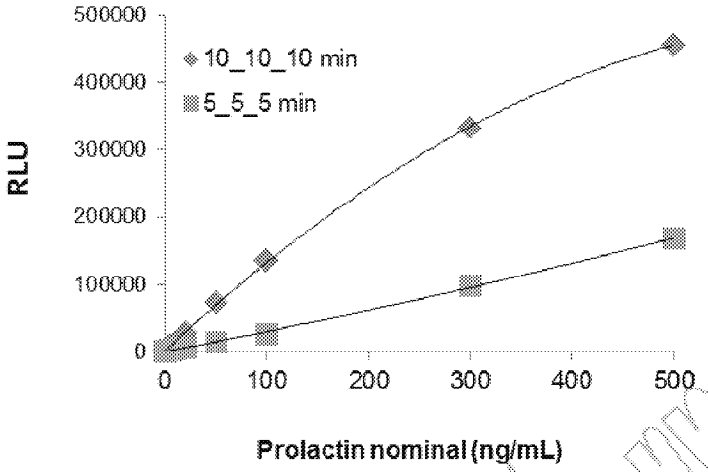


Table 13: Comparison of reagents incubation time

Prolactin Standard			10_10_10			5_5_5
ng/mL	Ave.RLU	CV	Modulation	Ave.RLU	CV	Modulation
500	455492	11.5	189.6	168116	9.8	173.9
300	332716	19.7	138.5	98041	16.5	101.4
100	134733	20.3	56.1	25864	5.2	26.8
50	71585	17.0	29.8	14010	14.9	14.5
20	27697	17.3	11.5	5537	20.8	5.7
10	13524	13.6	5.6	3867	13.4	4.0
5	8468	15.1	3.5	2431	10.4	2.5
0	2403	9.2	1.0	967	19.7	1.0

1. 12 Interfering Matrixes

Hemolyzed, icteric, lipemic and Rheumatoid factor positive (RF) serum samples were obtained from ProMedDx. The recovery of prolactin spiked into these potentially interfering matrixes was evaluated on the Theranos System. The assay did not show any significant interference from above mentioned samples, spike recovery was within 20% of nominal (Table 11).

Figure 13: Matrix effects

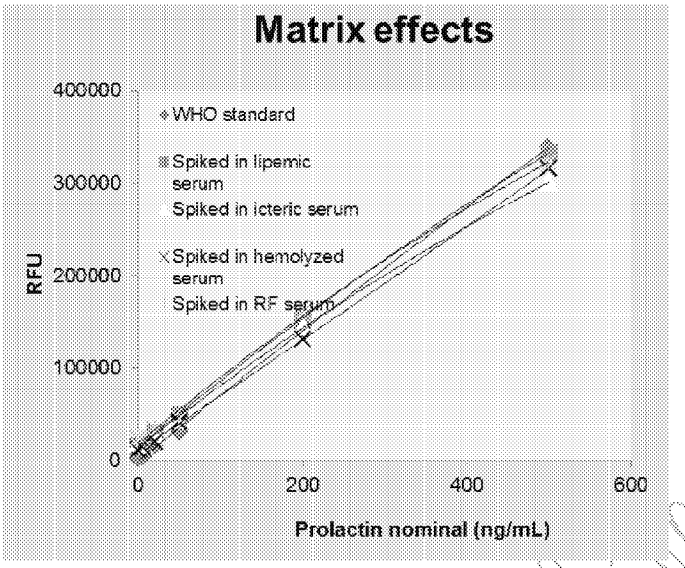


Table 14: Serum Spike Recovery

Prolactin ng/mL	% Recovery in lipemic	% Recovery in icteric	% Recovery in hemolyzed	% Recovery in RF serum
500	90.7	81.0	87.1	86.1
200	98.9	91.4	87.4	97.5
50	100.8	83.3	95.2	80.4
20	107.3	85.4	82.2	97.3
5	85.9	76.3	101.7	92.2
Ave.% Recovery	96.7	83.5	90.7	90.7

1. 13 Spike Recovery in EDTA-plasma versus Heparin-plasma

To determine whether prolactin has a similar spike recovery in EDTA-plasma vs. Heparin-plasma, paired plasma from 3 donors were tested. Four doses of prolactin were spiked and the spike recovery was calculated against the standard curve using calibrators prepared in serum. EDTA-plasma is a better matrix in spike recovery than Heparin-plasma, as shown in Table 12.

Table 15: Spike recovery in EDTA vs. Heparin plasma

Spike recovery	Donor I		Donor II		Donor III	
Prolactin	**EDTA**	**Heparin**	**EDTA**	**Heparin**	**EDTA**	**Heparin**
500 ng/mL	**95**	**75**	**95**	**73**	**90**	**73**
50 ng/mL	**82**	**71**	**88**	**79**	**70**	**60**
5 ng/mL	**69**	**68**	**97**	**98**	**83**	**74**
Ave. % Recovery	82	**72**	93	**84**	81	**69**

1.14 Determination of Expected LLOQ and ULOQ

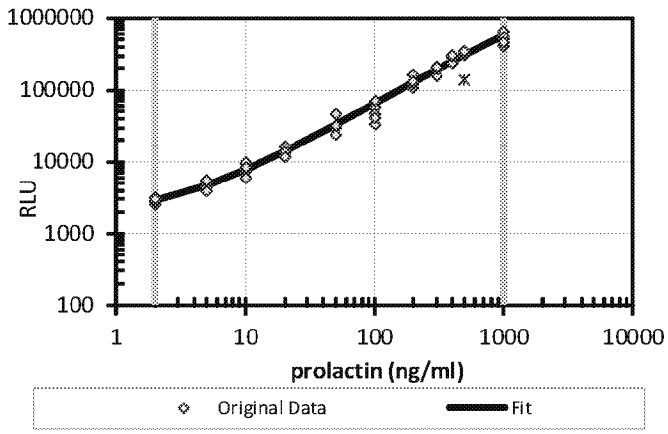
Prolactin serum calibration was performed on the final assay conditions of 25 ng/mL DAb in Biostab, 10 ug/mL CAb, and a 1:10 sample dilution with 3 cartridges per point. The LLOQ was 5 ng/mL and the ULOQ was 500 ng/mL.

Table 16: Serum Standard Curve for Determination of LLOQ and ULOQ

Prolactin (ng/mL)	Ave.RLU	CV	Modulation	Back-Calcu	Recovery
1000	521293	16	279.9	922.5	92.3
500	336001	5	180.4	521.7	104.3
400	288247	8	154.8	434.5	108.6
300	194239	9	104.3	280.1	93.4
200	135831	13	72.9	194.1	97.0
100	61115	20	32.8	90.7	90.7
50	34662	24	18.6	53.0	106.1
20	13698	12	7.4	20.0	99.9
10	8097	17	4.3	10.3	103.5
5	4703	11	2.5	4.7	93.0
2	2923	8	1.6	2.0	102.0
0	1862	14	1.0		

Conc = 10^(0.1771*(LOG(RLU))^3 -2.5476*(LOG(RLU))^2 + 13.149*(LOG(RLU)) - 22.034)

Figure 14: Serum Standard Curve for Determination of LLOQ and ULOQ

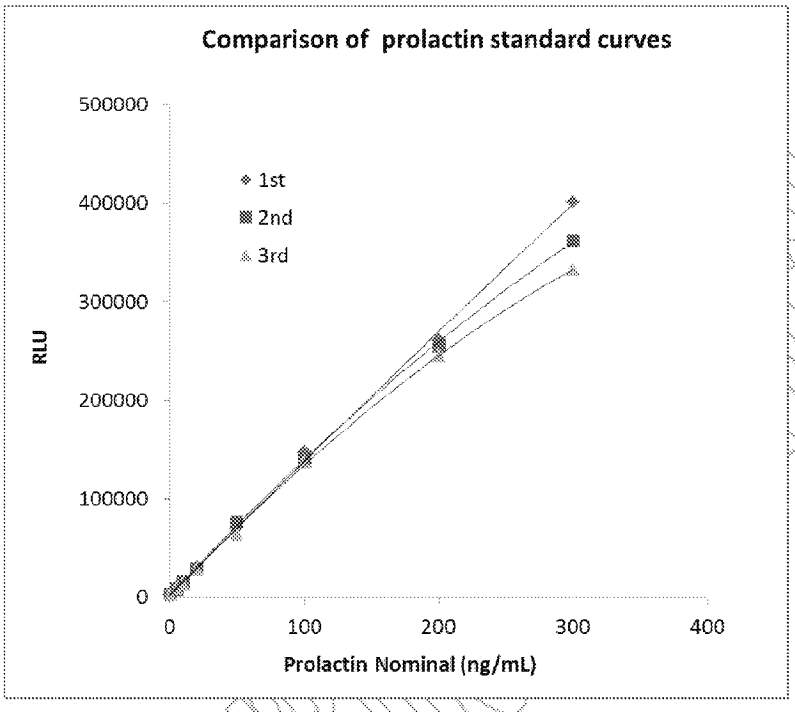


Summary		
Desired LLOQ	2	ng/mL
Desired ULOQ	1000	ng/mL
LLOQ accuracy	100.9	%
LLOQ precision	12.4	%
ULOQ accuracy	89.0	%
ULOQ precision	6.7	%

1.15 Precision test

Prolactin analyte in serum were run 3 times in the Theranos system with the final condition described above. The capture surfaces (Tips) for the 3 tests were from the same lot. The standard curves were aligned to each other with nice overlay.

Figure 15: Comparison of 3 standard curves



1.16 CV test

To test the variation in the Theranos system, two tips were taken from each of the 24 cartridges and selected for the CV test. Prolactin at 100 ng/mL was chosen for the test level. Both inter-, and intra-cartridges as well as total CVs were determined for the tips from the 24 cartridges. All the CVs determined were equal or less than 15%, at the acceptable level.

Table 17: CV test

	Prolactin 100 ng/mL in serum	Tip 1	Tip 2	Ave.	STDEV.	CV	Tip 1 Log5	Tip 2 Log5	Calculated 1	Calculated 2	Ave.	STDEV.	CV
Catridge1	'''	59543	49225	54384	7296	13	4.775	4.692	**88.6**	**73.7**	81.1	10.5	13.0
Catridge2	'''	67996	59551	63773	5972	9	4.832	4.775	**100.6**	**88.6**	94.6	8.5	9.0
Catridge3	'''	^102233	52505	52505		^49	#VALUE!	4.720		**78.4**	78.4		
Catridge4	'''	88613	86579	87596	1438	2	4.947	4.937	**129.6**	**126.8**	128.2	2.0	1.6
Catridge5	'''	69624	55056	62340	10302	17	4.843	4.741	**102.9**	**82.1**	92.5	14.7	15.9
Catridge6	'''	57459	73790	65625	11547	18	4.759	4.868	**85.6**	**108.8**	97.2	16.4	16.9
Catridge7	'''	69978	90434	80206	14464	18	4.845	4.956	**103.4**	**132.2**	117.8	20.3	17.3
Catridge8	'''	81338	82472	81905	801	1	4.910	4.916	**119.4**	**121.0**	120.2	1.1	0.9
Catridge9	'''	59954	58086	59020	1320	2	4.778	4.764	**89.1**	**86.5**	87.8	1.9	2.2
Catridge10	'''	84097	71313	77705	9039	12	4.925	4.853	**123.3**	**105.3**	114.3	12.7	11.1
Catridge11	'''	63800	67105	65452	2337	4	4.805	4.827	**94.6**	**99.3**	97.0	3.3	3.4
Catridge12	'''	83490	70389	76939	9264	12	4.922	4.848	**122.5**	**104.0**	113.2	13.1	11.5
Catridge13	'''	72208	74801	73504	1833	2	4.859	4.874	**106.6**	**110.2**	108.4	2.6	2.4
Catridge14	'''	61030	69127	65078	5725	9	4.786	4.840	**90.7**	**102.2**	96.4	8.2	8.5
Catridge15	'''	78958	79275	79116	224	0	4.897	4.899	**116.1**	**116.5**	116.3	0.3	0.3
Catridge16	'''	82752	70274	76513	8824	12	4.918	4.847	**121.4**	**103.8**	112.6	12.4	11.0
Catridge17	'''	79241	81995	80618	1947	2	4.899	4.914	**116.5**	**120.4**	118.4	2.7	2.3
Catridge18	'''	71026	^34534	71026		^45	4.851		**104.9**				
Catridge19	'''	90173	65728	77950	17285	22	4.955	4.818	**131.8**	**97.4**	114.6	24.4	21.3
Catridge20	'''	61901	74487	68194	8900	13	4.792	4.872	**91.9**	**109.8**	100.9	12.6	12.5
Catridge21	'''	72463	70071	71267	1691	2	4.860	4.846	**106.9**	**103.6**	105.2	2.4	2.3
Catridge22	'''	82041	58133	70087	16905	24	4.914	4.764	**120.4**	**86.5**	103.5	24.0	23.2
Catridge23	'''	68375	63814	66094	3225	5	4.835	4.805	**101.1**	**94.7**	97.9	4.6	4.7
Catridge24	'''	61273	58981	60127	1621	3	4.787	4.771	**91.0**	**87.7**	89.4	2.3	2.6

	Mean	STDEV	CV
Total CV	70664	10583	15
Inter CV	70664	9149	13
Intra CV			9

	Mean	STDEV	CV
Total CV	104	15	14
Inter CV	104	13	13
Intra CV			9

1.17 Shorter protocol test

Previously we have proved that a 5-5-5 min format is suitable for prolactin assay. In order to improve the time efficiency of our current prolactin ELISA in the Theranos system, several shortened protocols were further tested including 2-2-1 min with either 10 times sample dilution or 5 times sample dilution; in addition, coincubation formats were also selected, where final concentration of detection antibody is 2.5 ng/mL. All the formats tested were satisfactory and could result in very short running times.

Table 18: Protocol test

	10X (10-10-10 min)			10X (2-2-1 min)			5X (2-2-1 min)			Coincubation 10X (2-1 min)			Coincubation 10X (5-5 min)			Coincubation 25X (5-5 min)		
Prolactin	Mean	CV	Modulation	Mean	CV	Modulation	Mean	CV	Modulation	Mean	CV	Modulation	Mean	CV	Modulation	Mean	CV	Modulation
500	381821	16.9	186.8	18027	23.8	57.6	34372	20.7	108.8	64067	14.1	160.6	277016	14.6	188.6	22051	4.7	126.7
200	195352	11.5	95.6	7907	9.6	25.3	13857	9.8	43.9	31567	14.4	79.1	143626	5.1	97.8	8402	11.8	48.3
50	41289	12.8	20.2	1904	16.7	6.1	3421	8.8	10.8	6542	8.3	16.4	28137	12.6	19.2	1926	14.2	11.1
20	18453	5.2	9.0	857	7.6	2.7	1572	12.3	5.0	2561	10.4	6.4	11473	8.3	7.8	948	9.2	5.4
5	5201	18.7	2.5	447	19.3	1.4	556	15.6	1.8	966	17.9	2.4	3538	6.9	2.4	340	5.0	2.0
0	2044	11.9	1.0	313	13.0	1.0	316	8.6	1.0	399	9.5	1.0	1469	4.7	1.0	174	7.1	1.0

1.18 Stability test

We evaluated Cab stability by running on the Theranos system using Dab kept at 4 degrees. For Dab stability test, MTP plates were coated with fresh Cab each time to evaluate Dab stability at room temperature and 4 degrees. Cab is stable for the tested time period. However, Dab at room temperature lost about 50% of the activity. Therefore both Cab and Dab are recommended for 4-degree storage.

Figure 16: Cab stability

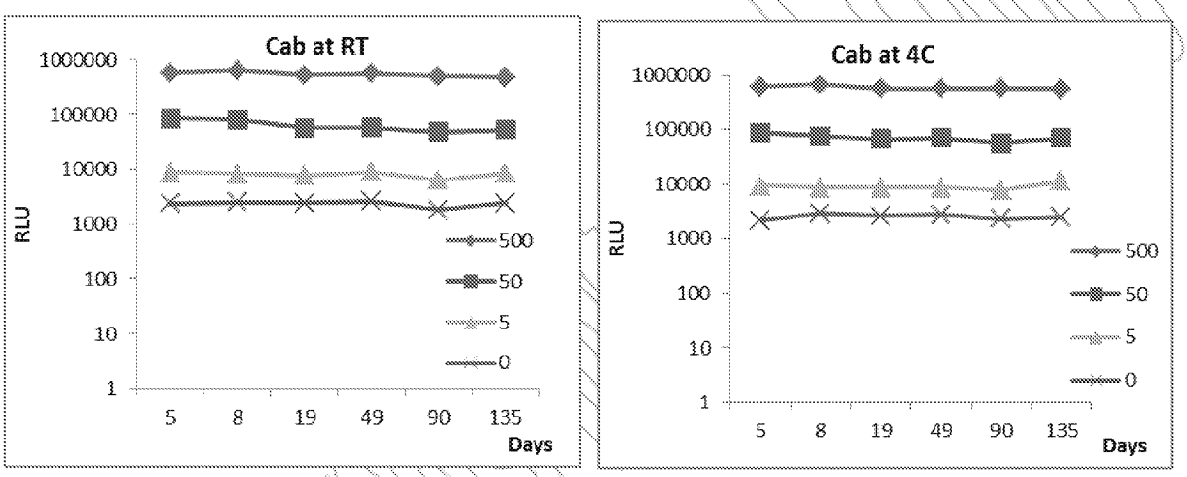


Figure 17: Dab stability