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Total Prostate-Specific Antigen (TPSA) Assay Development Report

Theranos Inc.

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1.1. Assay Specifications

Prostate-specific antigen (PSA) also known as gamma-seminoprotein or kallikrein-3 (KLK3) is a glycoprotein that in humans is encoded by the KLK3 gene. KLK3 is a member of the kallikrein-related peptidase family secreted by the epithelial cells of the prostate gland. PSA is often elevated in the presence of prostate cancer and in other prostate disorders. PSA exists in 3 major forms, free PSA, PSA-ACT (PSA bound to Alpha-1 Antichymotrypsin) and PSA-A2M (PSA bound to Alpha-2 Macroglobulin). Most PSA in the blood is bound to serum proteins. Total PSA level < 4 ng/mL in serum is considered low risk of prostate cancer. When TPSA levels are between 4 and 10 ng/mL, this is called grey zone. The ratio of free PSA to total PSA should be measured. The lower the ratio the greater is the probability of prostate cancer. Measuring the ratio of free to total PSA appears to be particularly promising for eliminating unnecessary biopsies in men for prostate cancer test.

The Theranos TPSA assay is designed to detect TPSA in human whole blood, plasma and serum. The assay has a reportable range of 0.0195 to 160 ng/mL, and is calibrated to the WHO international standard TPSA (90:10) (NIBSC, code 96/670). This assay has no cross-reactivity with kallikreins 2 and 4.

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

A commercial ELISA kit was used in house as predicate methods:

2. ASSAY DEVELOPMENT

2.1. Cross Reactivity and Interference (MTP)

Kallikrein 2 and 4 were tested for cross reactivity and interference with TPSA. Unacceptable interference was defined as greater than 120% or less than 80% of the controls.

In the cross-reactivity test, both kallikrein 2 (0 - 20 ng/mL) and kallikrein (0 - 200 ng/mL) has minimal reactions to TPSA antibody pair C7/D15. There is also no interference with TPSA signal when 50 ng/mL of kallikein 2 and 500 ng/mL of kallikrein 4 were tested.

Table 1: Cross Reactivity (MTP) Results

TPSA					KLK-2 (hk-2)					KLK-4				
ng/ml	1	2	Ave. RLU	CV	ng/ml	5	6	Ave. RLU	CV	ng/ml	9	10	Ave. RLU	CV
200	856317	855768	856043	0	20.00	19508	8898	14203	53	200	5855	6252	6054	5
50	247406	250273	248839	1	10.00	5988	5624	5806	4	50	5415	5130	5272	4
20	100853	99317	100085	1	5.00	7911	10582	9246	20	20	5841	5314	5578	7
5	33511	30516	32014	7	2.50	8126	7657	7891	4	5	6068	5412	5740	8
2	19413	14433	16923	21	1.25	6111	9576	7844	31	2	6313	4811	5562	19
0	10094	9015	9555	8	0.00	6255	4952	5604	16	0	4627	4544	4586	1

Table 2: Interference (MTP) Results

TPSA						
ng/ml	1	2	Ave. RLU	CV	Back-Calcula	% of Recovery
200	415198	432716	423957	3	203.29	101.64
50	124173	122419	123296	1	50.11	100.23
20	54088	58477	56283	6	20.90	104.51
5	17719	17713	17716	0	4.90	98.08
2	10693	10727	10710	0	2.06	103.09
0	5099	4798	4948	4	-0.26	
TPSA	**In the presence of KLK-2 (hk-2) 50 ng/ml**					
ng/ml	5	6	Ave. RLU	CV	Back-Calcula	% of Recovery
200	400057	403587	401822	1	190.78	95.39
50	120971	123073	122022	1	49.54	99.08
20	52480	58105	55292	7	20.48	102.42
5	18872	28223.5	18872	#DIV/0!	5.38	107.50
2	10935	14359.705	10935	#DIV/0!	2.15	107.63
0	6115	8108	7112	20	0.61	
TPSA	**In the presence of KLK-4 500 ng/ml**					
ng/ml	9	10	Ave. RLU	CV	Back-Calcula	% of Recovery
200	399006	431352	415179	6	198.30	99.15
50	124501	134705	129603	6	52.96	105.91
20	54786	57426	56106	3	20.83	104.13
5	18073	18070	18071	0	5.05	100.97
2	10278	10144	10211	1	1.86	93.00
0	4562	4637	4600	1	-0.40	

2.2. TPSA Standard Curve

The Theranos Analyzer test was completed with a regular serum standard curve to evaluate the antibody pair dose responses. TPSA calibrators were spiked in low TPSA serum screened by a commercial ELISA kit. The assay conditions were DAb 15 at 25 ng/mL in Stabilzyme, CAb 7 at 5 ug/mL in assay buffer and a sample dilution. A coincubation protocol with 5-5 min was chosen.

The selected pair C7/D15 showed a good dose response to TPSA ranging from 0.0195 to 400 ng/ml. It also has nice signal/background ratio (modulation) at the lower bottom of assay range. LLOQ obtained from Theranos software evaluation suggested that the detection limit of current assay is 0.0195 ng/mL.

Table 3: TPSA standard curve on Theranos Analyzer

tPSA ng/mL	Barcode	Reader	Tip1	Tip2	Intra Mean	Intra-CV	Inter mean	Inter CV	Modulation
400	927245593100100027	e000140	1333936	1310547	1322241	1	1178592	11	**3264.80**
	927245593100100028	e000071	1016522	1043082	1029802	2			
	927245593100100029	e000280	1181575	1185888	1183731	0			
160	927245593100100030	e000355	821359	743088	782223	7	796879	8	**2207.42**
	927245593100100031	e000327	776178	708995	742587	6			
	927245593100100032	e000178	865511	866146	865828	0			
40	927245593100100033	e000103	153842	188337	171089	14	203024	22	**562.39**
	927245593100100034	e000268	251869	263181	257525	3			
	927245593100100035	e000085	199141	161773	180457	15			
10	927245593100100036	e000188	80262	81712	80987	1	64155	23	**177.72**
	927245593100100037	e000075	62428	45907	54168	22			
	927245593100100038	e000337	52033	62591	57312	13			
2.5	927245593100100039	e000165	13765	11536	12651	12	13479	13	**37.34**
	927245593100100040	e000209	11058	14301	12680	18			
	927245593100100041	e000210	15448	14766	15107	3			
0.625	927245593100100042	e000272	4087	4176	4132	2	4088	9	**11.32**
	927245593100100043	e000126	4049	4106	4078	1			
	927245593100100044	e000124	3478	4632	4055	20			
0.156	927245593100100001	e000168	1405	1491	1448	4	1538	7	**4.26**
	927245593100100002	e000127	1671	1683	1677	0			
	927245593100100003	e000128	1460	1520	1490	3			
0.039	927245593100100045	e000284	731	719	725	1	713	12	**1.97**
	927245593100100046	e000278	1223	586	586	#DIV/0!			
	927245593100100047	e000328	824	705	764	11			
0.0195	927245593100100048	e000183	567	524	546	6	582	17	**1.61**
	927245593100100049	e000171	754	2588	754	#DIV/0!			
	927245593100100050	e000259	533	534	533	0			
0	927245593100100010	e000269	347	348	348	0	361	5	**1.00**
	927245593100100011	e000341	348	391	370	8			
	927245593100100012	e000163	362	371	366	2			

Figure [SEQ Figure * ARABIC]: TPSA standard curve on Theranos Analyzer

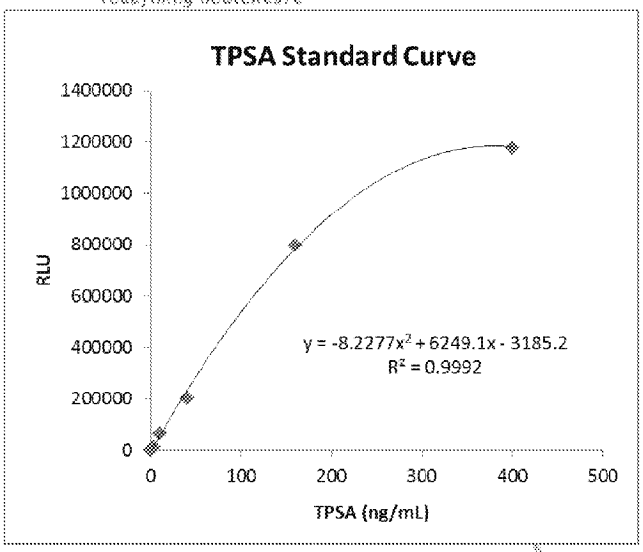
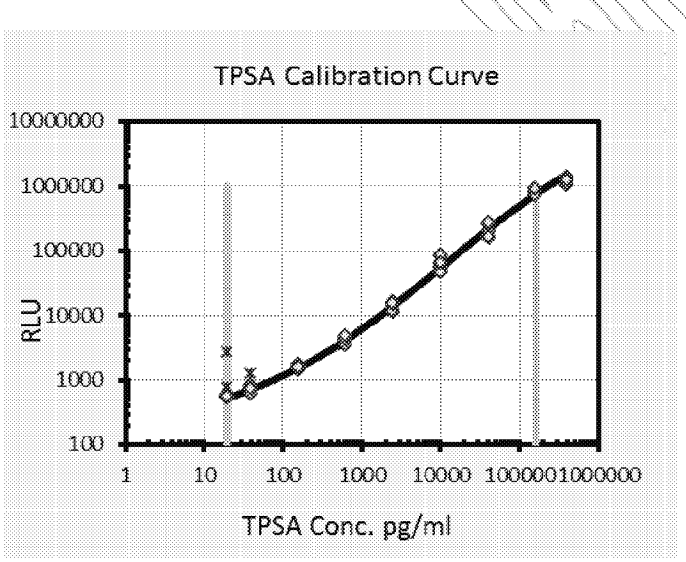


Figure 2: LLOQ and ULOQ (Theranos software1.0)

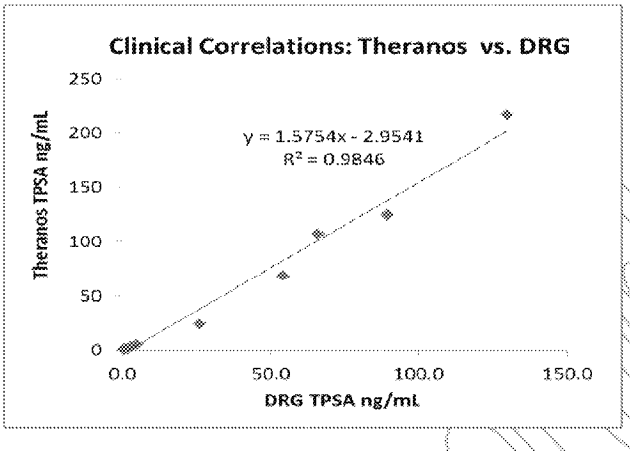


LLOQ	**19.50**	**pg/mL**
ULOQ	**160000.00**	**pg/mL**
desired LLOQ	**39.00**	**pg/mL**
desired ULOQ	**160000.00**	**pg/mL**
LLOD	**Not Reported**	**pg/mL**
LLOQ accuracy	**102**	**%**
LLOQ precision	**4.4**	**%**
ULOQ accuracy	**105**	**%**
ULOQ precision	**9.7**	**%**

2.3. Training Set

In order to evaluate if the antibody pair C7/D15 were able to track TPSA from patient samples, samples from Bioreclamationwere measured in the Theranos Analyzer as well as with DRG. The Theranos TPSA assay correlated closely with DRG ELISA, with a slope of 1.57 and R^2 of 0.985.

Figure 3: Training set—correlation with DRG kit

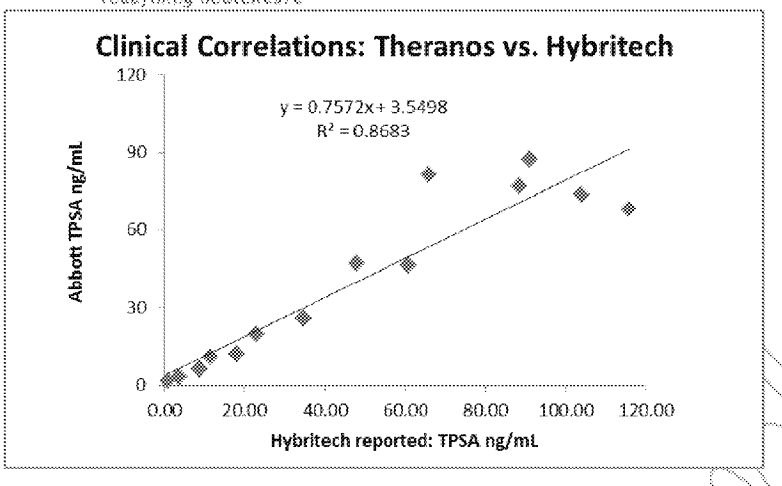


We further validated the Theranos TPSA assay with 15 specimens from Zeptometrix with reported TPSA concentrations. This PSA sensitivity panel contains 15 levels of TPSA. TPSA levels were pre-quantitated on clinical lab analyzers including Tosoh B, Hybritech and Ciba corning. TPSA concentration determined by the Theranos correlated very well with the reported TPSA values and resembled most with the Tosoh B assay.

Table 4: Clinical correlations (Zeptometrix TPSA sensitivity panel)

Samples	Theranos [tPSA] ng/mL	Tosoh B [tPSA] ng/mL	Hybritech [tPSA] ng/mL	Ciba Corning [tPSA] ng/mL
1	0.02	0.03		0.05
2	1.77	1.53	1.00	1.41
3	3.12	3.50	3.80	4.44
4	6.28	8.03	8.70	9.02
5	10.94	12.71	11.50	13.39
6	12.19	15.72	18.00	18.10
7	19.66	21.34	22.90	25.13
8	25.82	31.87	34.70	38.50
9	47.11	42.78	48.00	45.75
10	46.39	54.38	60.80	63.41
11	81.48	60.94	66.10	69.90
12	76.81	68.19	88.50	76.29
13	87.26	87.69	91.00	111.68
14	73.72	87.72	104.00	99.87
15	68.28	76.25	116.00	94.41

Figure 4: TPSA clinical correlations



2.4. Stabilizers Optimization for Detection Antibody

Stabilizer 2 and in-house blocking buffer (3% BSA TBS blocking buffer with 5 mM Mg and 0.1 mM Zinc) were tested for detection antibody stabilizers in the Theranos Analyzer in order to know if they could perform better than Stabilizer 1. It turned out that both stabilizer 1 and 2 are working well. At this time, we will stick with Stabilizer 1.

Table 5: Detection antibody stabilizers comparison

Dab 25 ng/ml	Stabilizer 1					Stabilizer 2					in-house blocking buffer				
TPSA, ng/ml	RLU1	RLU2	Mean	CV	Modulation	RLU1	RLU2	Mean	CV	Modulation	RLU1	RLU2	Mean	CV	Modulation
0.039	751	679	699	5	1.48	485	654	664	19	1.40	601	599	703	14	1.19
	713	659				836	692				762	824			
	688	707				654	13				771	659			
0.0195	610	662	575	18	1.22	598	657	609	6	1.28	428	604	520	12	0.88
	458	528				649	581				532	503			
	483	711				610	557				486	566			
0	493	494	471	8	1.00	379	413	474	25	1.00	607	655	592	7	1.00
	406	441				505	696				581	534			
	491	502				401	449				587	588			

2.5. Equimolarity

As previously mentioned, when TPSA levels are between 4 and 10 ng/mL, the ratio of free PSA to total PSA appears to be particularly promising for eliminating unnecessary biopsies in men for prostate cancer test. In order to monitor the effect of free PSA to total PSA, two sets of experiments were carried out. In the first set, we were going to find out if 3 ng/mL of TPSA would be interfered with an either 10%, 15% or 20% of free PSA in a serum free matrix. In the second set, we chose 10% of free PSA and added it to 1.5, 3, 5 and 10 ng/mL of TPSA. TPSA used in this assay is a mixture of 90% of complexed PSA and 10% of free PSA. The recovery of

TPSA in each setting is in a range of 90 -133% percent. This result is acceptable and considered as equimolarity. However, the result also suggested the more the free PSA used the more affect with TPSA recovery. Meanwhile, when fixed amount of free PSA was used, lower amount of TPSA was affected more than higher doses of TPSA.

Table 6: Effects of different amount of free PSA to 3 ng/mL of TPSA

Expected conc.	TPSA 3 ng/ml with free PSA	Ave. RLU	CV	Back-Calculated	% of recovery after correction
3.6	Free PSA 20%	22059	13.22	4.5	124.3
3.45	Free PSA 15%	19308	19.71	3.9	113.5
3.3	Free PSA 10%	18621	7.68	3.8	114.4

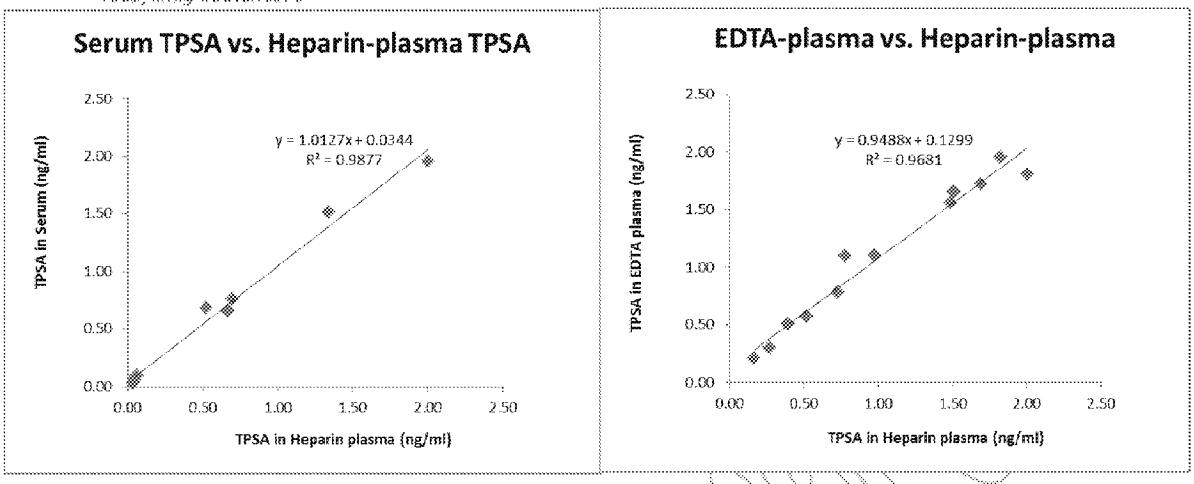
Table 7: Effects of 10% free PSA to TPSA from 1.5 to 10 ng/mL

Expected conc.	10% of free PSA with TPSA ng/ml	Ave. RLU	CV	Back-Calculated	% of recovery after correction
11	10	50618	16.22	10.3	93.3
5.5	5	31936	5.76	6.5	117.7
3.3	3	16763	11.95	3.4	103.0
1.65	1.5	10824	9.81	2.2	133.1

2.6. TPSA Detecting Ability in Serum, Heparin and EDTA plasma

EDTA-plasma was used for the development of the Theranos TPSA assay. Here, TPSA levels in matched serum and heparin plasma were compared. The average level of TPSA among the 9 serum samples was 0.6 ng/ml compared to a mean value of TPSA at 0.64 ng/ml with the matched heparin plasma samples. In addition, the two set of data showed a slope of 1.0127 and R^2 of 0.9877. This suggests that TPSA level maintains the same no matter serum or heparin plasma is used. Using the same method, TPSA level in 12 matched heparin- and EDTA-plasmas was also compared. The result expresses that the Theranos assay can use either EDTA- or heparin-plasma to measure TPSA because of a slope of 0.95 and R^2 of 0.968 in the correlation test.

Figure 5: TPSA level comparison in serum, heparin- and EDTA-plasma samples



2.7. Interfering Matrixes

Hemolyzed, icteric, lipemic, HAMA and rheumatoid factor positive (RF+) serum samples were obtained from ProMedDx. The recovery of TPSA spiked into these potentially interfering matrixes was evaluated in the Theranos Analyzer. The assay did not show any significant interference from the tested matrices, as the spike recovery was within 20% of nominal.

Table 8: TPSA recovery in hemolyzed serum samples

TPSA spiked in Hemolyzed serum #1					TPSA spiked in Hemolyzed serum #2				
tPSA ng/mL	Mean RLU	CV	Back-calculat	%Recovery	tPSA ng/mL	Mean RLU	CV	Back-calcı	%Recover
160	877896	9	181.21	113.0	160	731572	15	138.67	86.5
40	365996	8	54.71	135.4	40	263027	15	36.59	90.7
10	96036	5	12.07	116.1	10	78387	16	9.80	94.7
0.625	9384	14	1.14	111.2	0.625	8319	9	1.00	102.5
0.039	4519	11	0.49	112.4	0.039	3880	14	0.41	105.1
0	3714	14	0.39	#DIV/0!	0	3440	9	0.35	#DIV/0!

Table 9: More matrix effects

TPSA spiked in	Icteric serum		Lipimic serum		RF+ serum		HAMA serum	
tPSA ng/mL	Back-calculatec	%Recovery	Back-calcula	%Recovery	Back-calculati	%Recove	Back-calcı	%Recovery
160	165.53	103.4	196.69	122.4	190.07	118.6	166.57	103.7
40	36.31	90.7	41.04	100.8	41.35	102.8	43.79	107.8
10	8.78	87.7	11.46	106.9	13.07	127.6	10.86	102.1
0.625	0.61	94.7	1.32	98.5	1.04	120.1	1.19	94.9
0.039	0.05	89.3	0.73	95.6	0.24	86.1	0.61	91.2
0	0.02	#DIV/0!	0.72	#DIV/0!	0.24	#DIV/0!	0.63	#DIV/0!

2.8. Collection Method Comparison

Blood collected via venous draw and fingerstick were compared. The recovered plasmas were run on the Theranos assay. Among 8 samples, TPSA recovery in fingerstick (F) populations has an average of 1.47 ng/ml vs. 1.51 ng/ml in venous draw (V) subjects. The % of F/V is 97.5, suggesting blood samples collected with either of methods is suitable for the Theranos assay.

Table 10: Comparison of fingerstick and venous blood draw method

IDs	Fingerstick	Venous
	tPSA (ng/mL)	
E1	0.64	1
E2	1.89	2.3
E3	0.92	0.91
E4	1.76	1.78
M10	2.05	1.86
M11	**0.61**	**0.6**
M12	3.07	2.88
M14	0.84	0.75
ave. level	1.47	1.51
	% of F/V	**97.5**

3. CONCLUSION

We have successfully developed an immunoassay to detect total Prostate-specific antigen (PSA) in human serum and plasma.