



**Transferrin (TRF)
Assay Development Report**

Theranos, Inc.

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

Transferrins (TRFs) are iron-binding [HYPERLINK "http://en.wikipedia.org/wiki/Blood_plasma" \o "Blood plasma"] [HYPERLINK "http://en.wikipedia.org/wiki/Glycoprotein" \o "Glycoprotein"] that control the level of free [HYPERLINK "http://en.wikipedia.org/wiki/Iron" \o "Iron"] and in human, *TF* [HYPERLINK "http://en.wikipedia.org/wiki/Gene" \o "Gene"] encodes for TRFs. Transferrin [HYPERLINK "http://en.wikipedia.org/wiki/Glycoprotein" \o "Glycoprotein"] bind iron very tightly, but reversibly. Transferrin has a molecular weight of around 80 [HYPERLINK "http://en.wikipedia.org/wiki/Atomic_mass_unit" \o "Atomic mass unit"] and contains two specific high-affinity Fe (III) binding sites. When not bound to iron, it is known as "apotransferrin" (or [HYPERLINK "http://en.wikipedia.org/wiki/Apoprotein" \o "Apoprotein"]). When a transferrin protein loaded with iron encounters a [HYPERLINK "http://en.wikipedia.org/wiki/Transferrin_receptor" \o "Transferrin receptor"] on the surface of a cell, it binds to it and, as a consequence, is transported into the cell in a [HYPERLINK "http://en.wikipedia.org/wiki/Vesicle_%28biology%29" \o "Vesicle (biology)"] by [HYPERLINK "http://en.wikipedia.org/wiki/Receptor-mediated_endocytosis" \o "Receptor-mediated endocytosis"]. In humans, transferrin consists of a polypeptide chain containing 679 [HYPERLINK "http://en.wikipedia.org/wiki/Amino_acids" \o "Amino acids"]. The protein is composed of [HYPERLINK "http://en.wikipedia.org/wiki/Alpha_helix" \o "Alpha helix"] and [HYPERLINK "http://en.wikipedia.org/wiki/Beta_sheet" \o "Beta sheet"] to form two [HYPERLINK "http://en.wikipedia.org/wiki/Protein_domain" \o "Protein domain"]. The N- and C-terminal sequences are represented by globular lobes and between the two lobes is an iron-binding site. The [HYPERLINK "http://en.wikipedia.org/wiki/Amino_acids" \o "Amino acids"] which bind the iron ion to the transferrin are identical for both lobes; two [HYPERLINK "http://en.wikipedia.org/wiki/Tyrosines" \o "Tyrosines"], one [HYPERLINK "http://en.wikipedia.org/wiki/Histidine" \o "Histidine"], and one [HYPERLINK "http://en.wikipedia.org/wiki/Aspartic_acid" \o "Aspartic acid"]. For the iron ion to bind, an [HYPERLINK "http://en.wikipedia.org/wiki/Anion" \o "Anion"] is required, preferably [HYPERLINK "http://en.wikipedia.org/wiki/Carbonate" \o "Carbonate"] (CO_2^-).

Transferrin also has a transferrin iron-bound [HYPERLINK "http://en.wikipedia.org/wiki/Receptor_%28biochemistry%29" \o "Receptor (biochemistry)"]; it is a disulfide-linked [HYPERLINK "http://en.wikipedia.org/wiki/Homodimer" \o "Homodimer"]. In humans, each monomer consists of 760 amino acids. It enables [HYPERLINK "http://en.wikipedia.org/wiki/Ligand" \o "Ligand"] bonding to the transferrin, as each [HYPERLINK "http://en.wikipedia.org/wiki/Monomer" \o "Monomer"] can bind to one or two molecules of iron. Each monomer consists of three domains: the protease, the helical, and the apical domains. The shape of transferrin receptor resembles a butterfly-like complex, due to the shaped domains.

Transferrin imbalance can have serious health effects for those with low or high serum transferrin levels. Elevated levels of serum TRF are found in iron deficiency patients, women who use oral contraceptives, pregnancy, and hepatitis, for example. Depressed levels are found in acute inflammation, chronic liver disease, and hemochromatosis. An absence of transferrin results from a rare genetic disorder known as [[HYPERLINK "http://en.wikipedia.org/wiki/Atransferrinemia"](http://en.wikipedia.org/wiki/Atransferrinemia) \o "Atransferrinemia"]], a condition characterized by anemia and [[HYPERLINK "http://en.wikipedia.org/wiki/Hemosiderosis"](http://en.wikipedia.org/wiki/Hemosiderosis) \o "Hemosiderosis"] in the heart and liver that leads to many complications, including heart failure. Most recently, transferrin and its receptor have been shown to diminish [[HYPERLINK "http://en.wikipedia.org/wiki/Tumour_cells"](http://en.wikipedia.org/wiki/Tumour_cells) \o "Tumour cells"] by using the receptor to attract [[HYPERLINK "http://en.wikipedia.org/wiki/Antibodies"](http://en.wikipedia.org/wiki/Antibodies) \o "Antibodies"].

This quantitative assay is designed to detect Transferrin (TRF) in human whole blood, plasma, and serum. The assay has a reportable range of 4 to 577 mg/dL, and it was verified by using the IRMM Reference Material, catalog # ERM®-DA470k/IFCC as a “QC Control.”

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

Reference assay used was the TRF assay on the Siemens Advia 1800, and the method principle was polyethylene glycol (PEG) enhanced immunoturbidimetric.

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

Theranos TRF assay is designed as a sandwich ELISA. A biotin-labeled anti-TRF antibody is coated on an avidin surface and serves as the capture surface. The sample (whole blood, plasma or serum) is diluted and incubated for 2 minutes. Then an alkaline phosphatase-labeled anti-TRF secondary antibody acts as the detector and is incubated for 2 minutes. The surface is washed and the alkaline phosphatase substrate is incubated on the surface for 1 minute. The resulting chemiluminescence is read in Relative Light Units (RLUs).

Table [SEQ Table * ARABIC]: Materials

Reagent Name	Supplier	Catalog #
IRMM Reference Material	European Commission Joint Research Centre	ERM/IFCC-DA470K
TRF, Native Protein	Cell Sciences	CSI19828A
Purified Anti-Human TRF, Mab, (capture)	CalBioreagents	M352
Purified Anti-Human TRF, Mab (detector)	Lifespan Biosciences	LS-C39804/32235
Anti-TRF MAbs (Replacement detector in lieu of Lifespan Biosciences)	abcam	Ab10210
Alkaline Phosphatase Labeling Kit (SH)	Dojindo	LK13-10
Biotin Labeling Kit (SH)	Theranos	N/A
Theranos in-house AP Substrate	Theranos	N/A
Blocking Buffer (3% BSA/0.05% Sodium Azide in TBS)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G
Carbonate-bicarbonate buffer	Sigma	C3041

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

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

To determine the optimal pair for the TRF assay, all combinations of 20 TRF antibodies (Table 2) were tested on microtitre plates (MTPs). The screening was performed using Cell Sciences TRF, Native Protein (cat # CSI19828A) diluted in assay buffer, 10 ug/ml of capture and 100 ng/ml of detector in blocking buffer. The antibody screen is summarized in Table 3 and the data for the best antibody pairs with S/B ratio ≥ 400 that were chosen to proceed forward is summarized in Table 4. Specifically, the pairs selected were, as follow: C7/D2, C15/D1, C5/D11, C14/D4, C13/D12, C2/D13, C3/D12, C8/D15, and C19/C20.

Table [SEQ Table * ARABIC]: Antibody Information

Number	Vendor	Cat #	Host	Type
1	CalBioreangets	M351	N/A	M-Ab
2	CalBioreangets	M352	N/A	M-Ab
3	Novus Biologicals	NB110-16316	Goat	P-Ab
4	mybiosource	MBS311097	Mouse	M-Ab
5	mybiosource	MBS221751	Goat	P-Ab
6	Novus Biologicals	NBP1-78097	Rabbit	P-Ab
7	Novus Biologicals	NB110-7906	Mouse	M-Ab
8	Novus Biologicals	NB500-418	Mouse	M-Ab
9	Novus Biologicals	NBP1-44955	Mouse	M-Ab
10	Novus Biologicals	NB100-1947	Sheep	M-Ab
11	mybiosource	MBS311238	Mouse	M-Ab
12	Lifespan Biosciences	LS-C39787/32026	Mouse	M-Ab
13	Lifespan Biosciences	LS-C39804/32235	Mouse	M-Ab
14	Lifespan Biosciences	LS-C39789/32234	Mouse	M-Ab
15	Lifespan Biosciences	LS-C45708/17234	Mouse	M-Ab
16	Lifespan Biosciences	LS-C86319/31837	Goat	P-Ab
17	Lifespan Biosciences	LS-C85575/31835	Mouse	M-Ab
18	Lifespan Biosciences	LS-C83354/31759	Mouse	M-Ab
19	Lifespan Biosciences	LS-C62095/31880	Mouse	M-Ab
20	Lifespan Biosciences	LS-C122493/32049	Mouse	M-Ab

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

D-Ab	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16	#17	#18	#19	#20
C-Ab	#1																			
#1																				
#2																				
#3																				
#4																				
#5																				
#6																				
#7																				
#8																				
#9																				
#10																				
#11																				
#12																				
#13																				
#14																				
#15																				
#16																				
#17																				
#18																				
#19																				
#20																				

Antibodies screened should be coded so as not to give away clone number throughout file.

 Excellent dose response: S/B >400, and/or background is < 1000; final picked pairs

 Good dose response, S/B >300

 Medium dose response, S/B ≥ 100, and/or high background

 Little/no dose response, S/B <100, and/or high background

 Not determined = Nd

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

Ab pair: C7D2						Ab pair: C15D1						Ab pair: C5D11						Ab pair: C7D2						Ab pair: C15D1											
[Transferrin]		Nominal mg/dL		Values		MeanValue		Std.Dev.		CV%		S/B		Values		MeanValue		Std.Dev.		CV%		S/B		Values		MeanValue		Std.Dev.		CV%		S/B			
500	982566	879056	146385	17	1582									908172	881774	37333	4	1457					937211	938878	2357	0	1869								
	775547													855376									940545												
100	316059	300819	21553	7	541									481781	465137	23538	5	769					352919	343819	12870	4	684								
	285579													448493									334718												
10	16708	16543	233	1	30									63012	63401	550	1	105					25385	25880	699	3	52								
	16378													63789									26374												
0	460	556	135	24										577	605	40	7					439	502	90	18										
	651													633									566												
[Transferrin]						Ab pair: C14D4						Ab pair: C13D12						Ab pair: C2D13						Ab pair: C3D12						Ab pair: C8D15					
[Transferrin]		Nominal mg/dL		Values		MeanValue		Std.Dev.		CV%		S/B		Values		MeanValue		Std.Dev.		CV%		S/B		Values		MeanValue		Std.Dev.		CV%		S/B			
500	1123990	1102382	30559	3	1272									1374463	1325942	68619	5	1390					705584	641154	91118	14	474								
	1080774													1277421									576724												
100	922320	852106	99299	12	983									715703	721792	8612	1	757					139634	128962	15092	12	95								
	781891													727881									118290												
10	176054	167658	11875	7	193									100828	98014	3980	4	103					9134	8468	942	11	6								
	159261													95200									7802												
0	783	867	119	14										1022	954	96	10					1374	1353	29	2										
	951													886									1332												
[Transferrin]						Ab pair: C19D20						Ab pair: C19D20						Ab pair: C19D20						Ab pair: C19D20						Ab pair: C19D20					
[Transferrin]		Nominal mg/dL		Values		MeanValue		Std.Dev.		CV%		S/B		Values		MeanValue		Std.Dev.		CV%		S/B		Values		MeanValue		Std.Dev.		CV%		S/B			
500	645745	654956	13025	2	609									89472	83980	7767	9	47					103826	106939	4402	4	220								
	664166													78488									110052												
100	273002	281016	11333	4	261									13427	16168	3877	24	9					27926	28571	913	3	59								
	289029													18910									29217												
10	28498	27627	1232	4	26									3546	3310	334	10	2					2972	3010	54	2	6								
	26755													3074									3049												
0	700	1075	530	49										1525	1790	375	21					369	485	118	24										
	1450													2055									402												

2.02 Cross Reactivity and Interference (MTP)

Initially, cross reactivity and interference tests were conducted on microtitre plates (MTPs), and ferritin, human hemoglobin (hHb), and alpha-fetoprotein (αFP) were chosen for both cross reactivity test and interference test. Since TRF clinical range is measured in milligrams per deciliters (mg/dL), and the control curve was ran on MTPs at this concentrated unit of measurement, and it was observed that data from MTPs were too saturated and hence, unreliable. As a result, cross reactivity and interference tests were carried out on Theranos TRF Assay System. The ranges of the analytes tested for cross-reactivity were Ferritin at 0-1000 ng/mL; αFP at 0 – 20 ng/mL; and hHb at 0 – 18 g/dL. Additionally, acceptance criteria were defined for cross reactivity, that is, if the mean counts of each of the potential cross reactants were less than the counts of TRF at 0 mg/dL, then the potential cross reactant was deemed non-reactive. It was found that no cross reactivity was observed in Ab pairs C5/D11 and C2/D13.

One of the challenges in developing the TRF assay was to find a “good” Ab pair that would not interfere with similar proteins. With this in mind, the concentrations of the potential interfering proteins were adjusted for the interference test. For example, for Ab pair C5/D11, TRF standard

curve was spiked with 3x the highest calibrator for ferritin at 3000 ng/mL and α FP at 60 ng/mL; while hHb was not tested for interference since a more concentrated form of hHb was not available. And for Ab pair C2/D13, and for reasons previously mentioned and of clinical Relevance; TRF standard curve was spiked with 1.5x the highest calibrator for ferritin at 1500 and α FP at 20 ng/mL; while hHb was not tested for interference since a more concentrated form of hHb was not available. Unacceptable interference was defined as greater than 120% or less than 80% of the TRF control. No interference was observed for both Ab pairs C5/D11 and C2/D13, at the stated test concentrations. However, during further developmental steps, it was found that C5/D11 showed no low end modulation and hence, it was dropped from further developmental steps. Now, the only best Ab pair was C2/D13 and as a result, it was selected to continue with assay optimization, starting with "Capture Antibody Titration" Test.

The assay conditions used in the above tests were with TRF capture at 10 μ g/mL in Blocking Buffer with sodium azide in TBS (and from now on, will be referred to as Blocking Buffer); detector at 100 ng/mL in Blocking Buffer; and the TRF calibrators (Cell Sciences) were serially diluted in 3% BSA, 0.05 % Sodium Azide in TBS (and from now on, will be referred to as Assay Buffer).

Table 5: TRF Standard Curve in Assay Buffer for Ab pair C5/D11

[Transferrin] In sample mg/dL	[Transferrin] SIEMENS_ADVIA mg/dL	Signal (RLU): Inter-Cartridge			Back-calculated conc, mg/dL log-log fit			Inter-Cartridge Mean	% Recovery
		Mean	%CV	S/B	log Signal	log conc			
500	425	92934	20	295.3	4.97	2.63	446	25	105
300	263	57423	17	182.5	4.76	2.42	243	19	93
100	89	21690	23	68.9	4.34	1.95	94	20	106
50	44	8665	13	27.5	3.94	1.64	43	12	98
10	9	1998	17	6.4	3.30	0.95	9	23	97
5	5	1398	25	4.4	3.15	0.70	5	40	103
1	1	553	26	1.8	2.74		1	55	84
0	0	315	17				0		

Figure 1: TRF Calibration Curve in Assay Buffer for Ab pair C5/D11

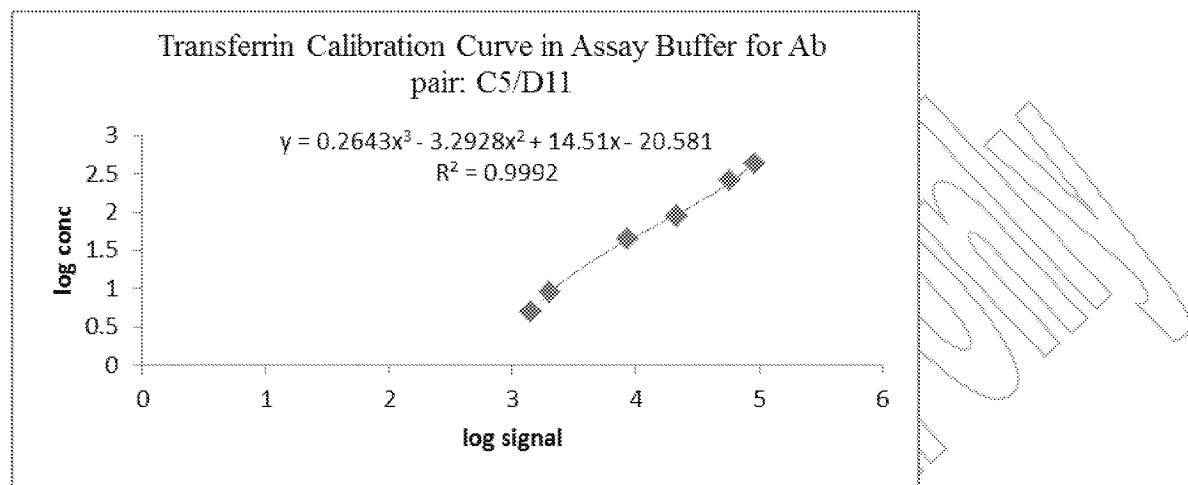


Table 6: Cross Reactivity (Theranos TRF Assay System) Results for Ab pair: C5/D11

[Ferritin] Nominal, ng/ml	[Ferritin] Reassigned, ng/ml	Signal (RLU)		Back-calc, ng/ml	
		Inter Mean	%CV	Inter Mean	% X Reactivity
1000	1351	343	20.22	0.24	0.06
500	613	306	22.24	0.18	0.07
100	117	278	18.73	0.13	0.14
50	57	351	41.84	0.31	3.42
10	9	300	11.76	0.15	3.08
0	0	430	39.80	2.25	
[hHb] Nominal, g/dL	[hHb] Reassigned, g/dL	Signal (RLU)		Back-calc, ng/ml	
		Inter Mean	%CV	Inter Mean	% X Reactivity
18	N/d	478	17.26	0.57	0.13
10	N/d	372	21.38	0.30	3.37
5	N/d	297	13.89	0.15	3.02
0	N/d	257	28.72	0.11	
[AFP] Nominal, ng/ml	[AFP] Reassigned, ng/ml	Signal (RLU)		Back-calc, ng/ml	
		Inter Mean	%CV	Inter Mean	% X Reactivity
20	N/d	221	20.12	0.06	0.01
10	N/d	206	23.39	0.05	0.02
5	N/d	354	85.73	1.10	1.24
2	N/d	204	21.25	0.05	0.54
1	N/d	206	18.21	0.05	0.96
0	N/d	218	29.12	4.56	

N/d denotes Not determined

Table 7: TRF Standard Curve in Assay Buffer for Ab pair C2/D13

[Transferrin] In sample mg/dL	[Transferrin] SIEMENS_ADVIA mg/dL	Signal (RLU): Inter-Cartridge			Back-calculated conc, mg/dL			% Recovery	
		Mean	%CV	S/B	log Signal	log conc	Mean		
500	425	20212	13	51	4.31	2.63	439	13	103
300	263	10680	20	27	4.03	2.42	254	14	97
100	89	2593	20	6.5	3.41	1.95	93	17	105
50	44	1272	21	3.2	3.10	1.64	43	29	98
10	9	525	16	1.3	2.72	0.95	9	36	95
5	5	440	24	1.1	2.64	0.70	6	58	115
1	1	462	16	1.2	2.66				
0	0	396	29						

Figure 2: TRF Calibration Curve in Assay Buffer for Ab pair C2/D13

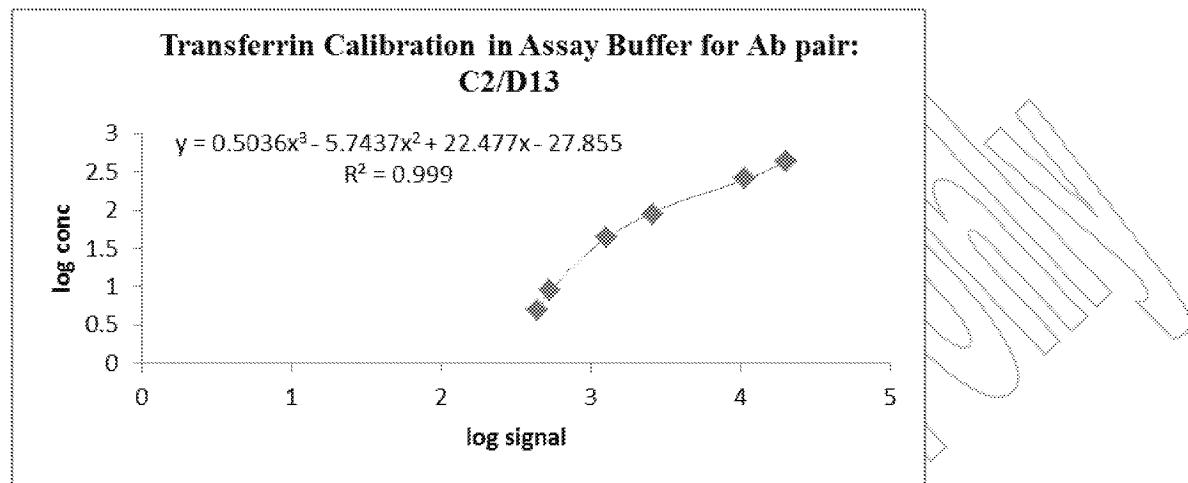


Table 8: Cross Reactivity (Theranos TRF Assay System) Results for Ab pair: C2/D13

[Ferritin] Nominal, ng/ml	[Ferritin] Reassigned, ng/ml	RLU		Back-calc, ng/ml	
		Inter Mean	%CV	Inter Mean	% X Reactivity
1000	1351	421	23	5.03	1.18
500	613	393	11	3.99	1.52
100	117	320	25	2.38	2.67
50	57	431	8	5.11	56.73
10	9	374	14	3.50	70.02
0	0	407	21	4.58	
[hHb] Nominal, g/dL	[hHb] Reassigned, g/dL	RLU		Back-calc, ng/ml	
		Inter Mean	%CV	Inter Mean	% X Reactivity
18	N/d	469	7	5.31	1.25
10	N/d	413	12	4.58	---
5	N/d	426	24	5.25	---
0	N/d	402	25		
[AFP] Nominal, ng/ml	[AFP] Reassigned, ng/ml	RLU		Back-calc, ng/ml	
		Inter Mean	%CV	Inter Mean	% X Reactivity
20	N/d	459	18	6.20	1.46
10	N/d	534	32	9.24	3.51
5	N/d	485	14	7.01	7.87
2	N/d	496	8	6.11	
1	N/d	442	14	3.71	
0	N/d	451	28	6.21	

N/d denotes Not determined

Table 9: Interference (Theranos TRF Assay System) Results for Ab pair: C5/D11

		Ferritin, 3x highest calibrator, which equated to 3000 ng/ml						
[Transferrin] In sample mg/dL	[Transferrin] SIEMENS ADVIA mg/dL	RLUs: Inter-Cartridge			Back-calc, mg/dL			
		Mean	%CV	Inter Mean	%CV	% from Control		
500	425	107335	19.6	548	27.6	129		
300	263	56814	19.8	241	23.1	92		
100	89	23151	28.9	99	25.6	112		
10	9	2382	23.4	11	30.8	123		
5	5	1257	19.0	4	31.7	86		
0	0	343	29.0	0	103.2			

		AFP, 3x highest calibrator, which equated to 60 ng/ml						
[Transferrin] In sample mg/dL	[Transferrin] SIEMENS ADVIA mg/dL	RLUs: Inter-Cartridge			Back-calc, mg/dL			
		Mean	%CV	Inter Mean	%CV	% from Control		
500	425	92396	33.5	457.85	44.3	108		
300	263	49593	43.6	213.11	45.9	81		
100	89	23497	18.4	100.77	40.3	113		
10	9	2245	49.6	10.20	59.1	113		
5	5	1320	18.1	4.65	50.4	93		
0	0	302	16.2	0.16	54.4			

Table 10: Interference (Theranos TRF Assay System) Results for Ab pair: C2/D13

[Transferrin] In sample mg/dL	[Transferrin] SIEMENS ADVIA mg/dL	Ferritin at 1500 ng/ml					Ferritin at 1000 ng/ml				
		Signal (RLUs)		Back-calculated conc, mg/dL			Signal (RLUs)		Back-calculated conc, mg/dL		
		Inter Mean	%CV	Inter Mean	%CV	% from Control	Inter Mean	%CV	Inter Mean	%CV	% from Control
500	425	23296	23	519	27	122	23483	21	522	24	123
300	263	9331	23	202	39	77	11122	26	263	20	100
100	89	3754	17	126	12	141	3232	10	113	7	127
10	9	419	18	5	47	54	452	14	6	37	65
5	5	332	9	2	26	48	362	21	3	58	67
0	0	303	15	2	48		291	19	2	71	
[Transferrin] In sample mg/dL	[Transferrin] SIEMENS ADVIA mg/dL	AFP at 20 ng/ml					AFP at 10 ng/ml				
		Signal (RLUs)		Back-calculated conc, mg/dL			Signal (RLUs)		Back-calculated conc, mg/dL		
		Inter Mean	%CV	Inter Mean	%CV	% from Control	Inter Mean	%CV	Inter Mean	%CV	% from Control
500	425	27294	12	619	16	146	25993	22	590	26	139
300	263	13048	26	297	21	113	11089	21	249	19	95
100	89	2819	29	124	52	139	3057	15	108	12	121
10	9	375	9	3	26	39	436	11	5	30	59
5	5	316	11	2	35	42	364	7	3	21	63
0	0	272	24	1			281	11	1	37	

2.03 Capture (C2) Antibody Titration

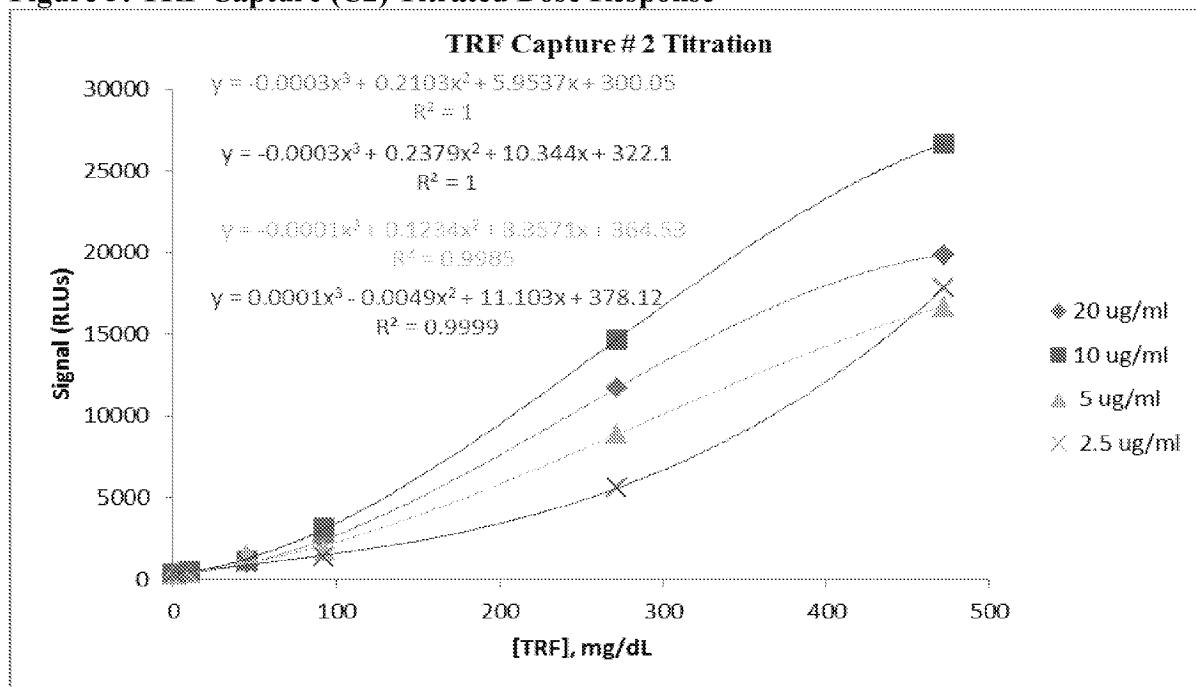
The capture antibody (C2) was titrated at 20, 10, 5, and 2.5 ug/mL in Blocking Buffer. The detector antibody (D13) concentration used was at 100 ng/mL in Blocking Buffer with a sample dilution of 1:10000x. The TRF (Cell Sciences) calibrators were prepared in Assay Buffer. Results showed that the capture antibody at 10 ug/mL yielded the best signal over background (S/B) ratio and excellent modulation at the lower bottom end of the standard curve. 10 ug/ml was finalized as the capture Ab concentration for the TRF assay.

Table 11: Capture (C2) Ab Titration Results

[Transferrin] In sample mg/dL	[Transferrin] SIEMENS ADVIA mg/dL	20 ug/mL			10 ug/mL		
		Signal (RLU) Inter-Cartridge			Signal (RLU) Inter-Cartridge		
		Mean	%CV	S/B	Mean	%CV	S/B
500	472	19842	33	65.8	26654	17	75.6
300	272	11718	35	38.9	14688	18	41.6
100	92	2371	32	7.9	3124	20	8.9
50	45	1009	12	3.3	1133	13	3.2
10	10	451	26	1.5	471	16	1.3
5	5	252	15	0.8	385	26	1.1
1	1	306	32	1.0	320	26	0.9
0	0	302	15		353	35	

[Transferrin] In sample mg/dL	[Transferrin] SIEMENS ADVIA mg/dL	5 ug/mL			2.5 ug/ml		
		Signal (RLU) Inter-Cartridge			Signal (RLU) Inter-Cartridge		
		Mean	%CV	S/B	Mean	%CV	S/B
500	472	16710	28	55.4	17808	10	49.6
300	272	8923	19	29.6	5589	57	15.6
100	92	1738	41	5.8	1372	27	3.8
50	45	1471	47	4.9	1000	35	2.8
10	10	362	20	1.2	517	36	1.4
5	5	476	31	1.6	396	16	1.3
1	1	283	13	0.9	373	22	1.0
0	0	285	13		359	17	

Figure 3: TRF Capture (C2) Titrated Dose Response



2.04 Sample Dilution

From the “Capture Titration” Experiment, it was observed that although there was relatively good modulation, however, the signals (RLUs) were also relatively low. A sample dilution test was conducted and found that a 5000x versus 10000x sample dilution afforded us with higher signals (RLUs) and better low end modulation.

Table 12: Sample Dilution (5000x) Results

[Transferrin] In sample mg/dL	[Transferrin] SIEMENS ADVIA mg/dL	Signal (RLU) Inter-Cartridge			[Back-calculated], mg/dL		
					Inter-Cartridge		% Recovery
		Mean	%CV	S/B	Mean	%CV	
500	472	135201	16	493.6	516	21	109
300	272	63705	18	232.6	235	16	86
100	92	16798	10	61.3	94	6	102
50	45	5700	13	20.8	49	9	110
10	10	1128	7	4.1	11	10	106
5	5	599	18	2.2	4	32	79
1	1	329	25	1.2	1	55	121
0	0	274	15		1		

2.05 Detector (D13) Antibody Titration

Detection antibody (D13) was titrated at 200, 100, 50, and 25 ng/mL, to find the optimal working concentration. In this test, TRF capture at 10 ug/mL in Blocking Buffer; TRF calibrators (Cell Sciences) were serially diluted in Assay Buffer; and the sample dilution was 1:5000x. The D-Ab concentration of 100 ng/ml gave the best modulation across the standard curve, and in particular at the lower end of the assay where high sensitivity was desired with excellent low background. Therefore, 100 ng/ml was selected for detection antibody (D13) as the final working concentration (Table 13, Figure 4).

Then, the detection antibody was further tested with stabilizers such as Theranos in-house AP-Stabilizer with magnesium and zinc; Biostab Stabilizer; and StabilZyme AP. Based on the results, the Ab pair C2/D13 performed best in Theranos in-house AP-Stabilizer with magnesium and zinc and thus, it was selected as the stabilizer of choice (Table 14, Figure 6).

Table 13: TRF Detector Ab Titration Results

[Transferrin] In sample mg/dL		[Transferrin] SIEMENS_ADVIA mg/dL		Detector at 200 ng/ml Signal (RLU) Inter-Cartridge			Detector at 100 ng/ml Signal (RLU) Inter-Cartridge		
		Mean	%CV	S/B		Mean	%CV	S/B	
750	577	362374	11	516.3	237281	26	423.0		
500	388	258818	10	368.8	162177	29	289.1		
400	313	167937	27	239.3	120085	19	214.1		
300	238	149025	28	212.3	113265	20	201.9		
200	156	68287	35	97.3	63153	11	112.6		
100	79	37599	24	53.6	28247	11	50.4		
50	39	13318	10	19.0	9917	8	17.7		
10	7	2288	15	3.3	1763	19	3.1		
0	0	702	24		561	36			

[Transferrin] In sample mg/dL		[Transferrin] SIEMENS_ADVIA mg/dL		Detector at 50 ng/ml Signal (RLU) Inter-Cartridge			Detector at 25 ng/ml Signal (RLU) Inter-Cartridge		
		Mean	%CV	S/B		Mean	%CV	S/B	
750	577	106970	18	399.1	61953	20	245.5		
500	388	74448	18	277.8	46384	21	183.8		
400	313	49394	19	184.3	34165	21	135.4		
300	238	41774	22	155.9	30524	17	121.0		
200	156	24933	11	93.0	16864	11	66.8		
100	79	11093	44	41.4	6236	9	24.7		
50	39	3407	14	12.7	2295	15	9.1		
10	7	521	24	1.9	413	14	1.6		
0	0	268	30	1.0	252	18			

Figure 4: TRF Detector (D13) Titrated Dose Response

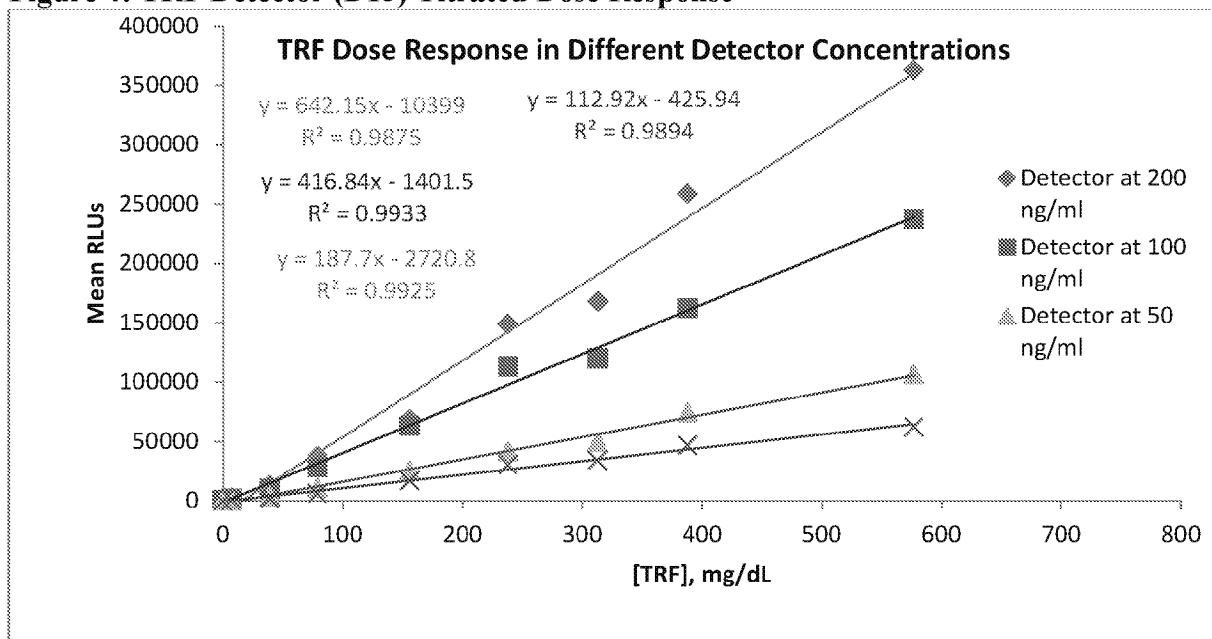
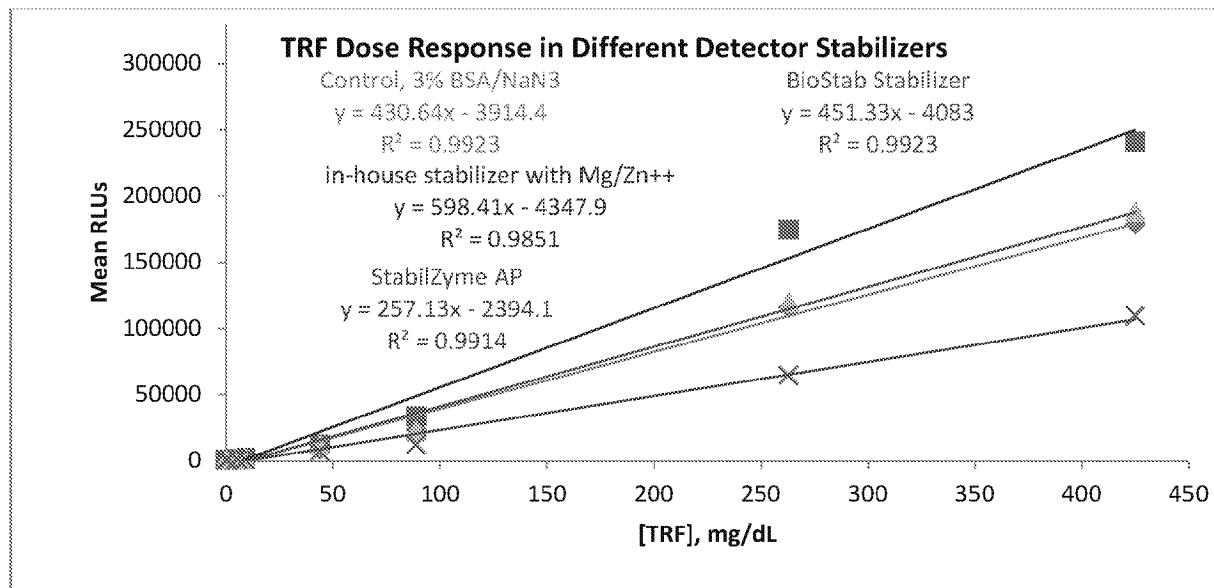


Table 14: TRF Stabilizers Results

[Transferrin] In sample mg/dL	[Transferrin] SIEMENS_ADVIA mg/dL	Detector Stabilizer: 3% BSA/NaN3 Signal (RLU) Inter-Cartridge			Detector Stabilizer: Theranos in-house stabilizer with Mg++/Zn++ Signal (RLU) Inter-Cartridge		
		Mean	%CV	S/B	Mean	%CV	S/B
500	425	178639	18	362.1	240956	5	363.9
300	263	114903	23	232.9	174434	5	263.4
100	89	23127	23	46.9	33221	23	50.2
50	44	8460	26	17.1	12316	9	18.6
10	9	1412	24	2.9	1989	9	3.0
5	5	1083	19	2.2	1210	15	1.8
1	1	585	26	1.2	696	10	1.1
0	0	493	21		662	21	

[Transferrin] In sample mg/dL	[Transferrin] SIEMENS_ADVIA mg/dL	Detector Stabilizer: BioStab Signal (RLU) Inter-Cartridge			Detector Stabilizer: StabilZyme AP Signal (RLU) Inter-Cartridge		
		Mean	%CV	S/B	Mean	%CV	S/B
500	425	188156	9	327.2	109203	12	206.1
300	263	119138	14	207.2	64529	25	121.8
100	89	22404	37	39.0	12197	21	23.0
50	44	11651	16	20.3	6975	19	13.2
10	9	1210	23	2.1	1103	20	2.1
5	5	974	14	1.7	774	18	1.5
1	1	538	18	0.9	494	11	0.9
		575	18		530	9	

Figure 5: TRF Detector Stabilizers Dose Responses



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2.06 Effect of Coating Buffers

Four different coating buffers: 3% BSA (Control), Starting Block, Sea Block, and Superblock were tested to see which one would give us the highest signal to background (S/B) ratio and sensitivity. In this test, detection antibody (D13) was at 100 ng/ml in Theranos in-house AP-Stabilizer with magnesium and zinc and sample dilution was 1:5000x. It was found that 3% BSA (Control) yielded the highest S/B and sensitivity, and thus, it was selected as the coating buffer (Figure 6).

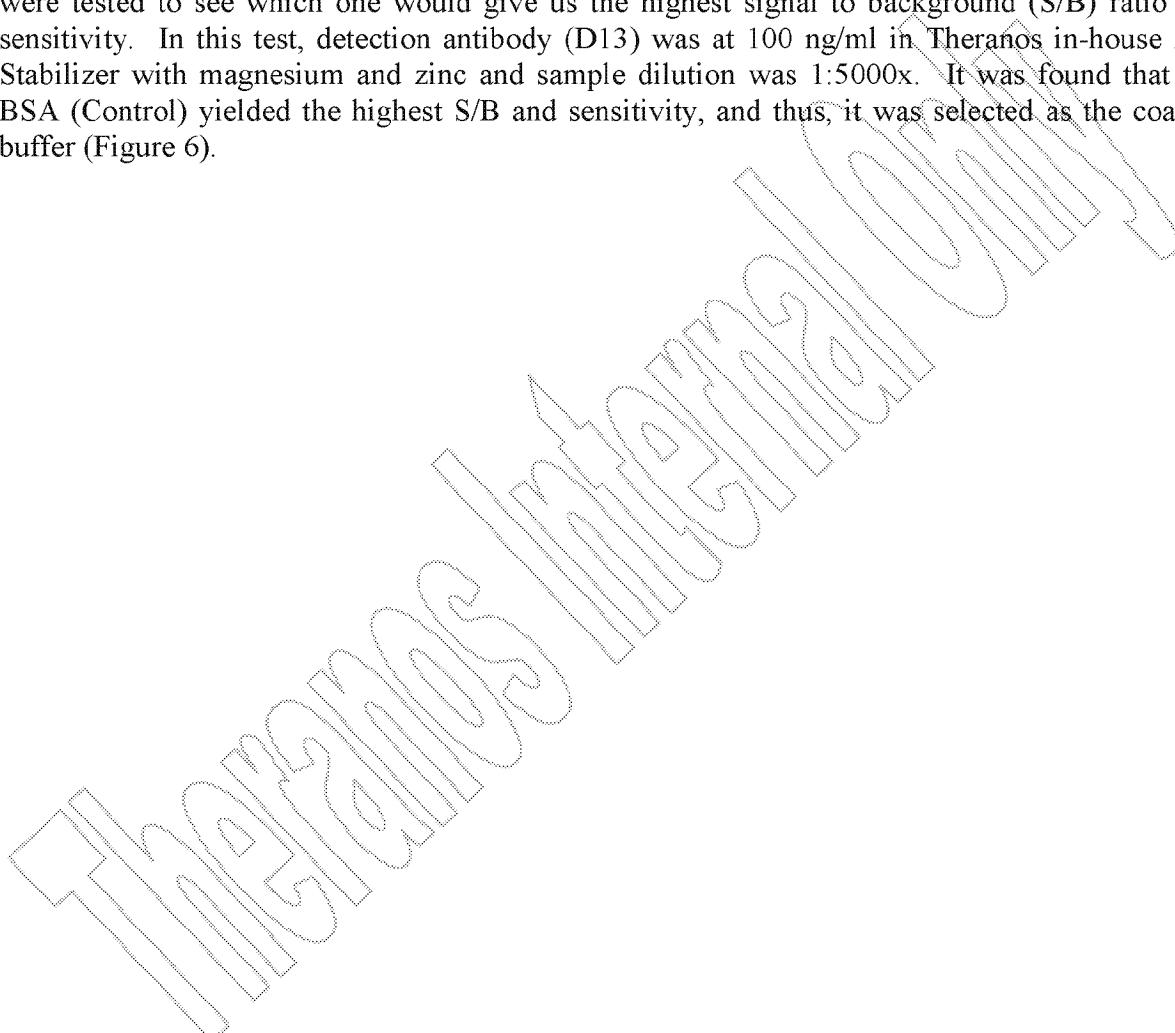


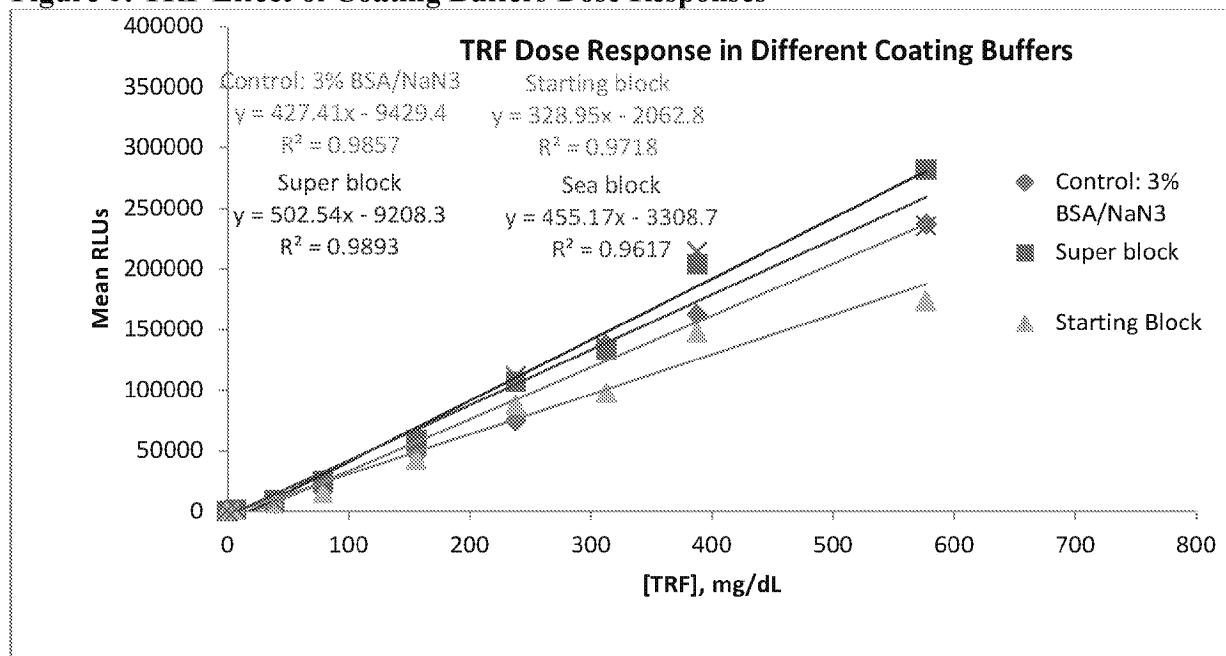
Table 15: TRF Effect Coating Buffers Results

[Transferrin] In sample mg/dL	[Transferrin] SIEMENS ADVIA mg/dL	Control: 3% BSA/NaN3 Signal (RLU) Inter-Cartridge			Super block Signal (RLU) Inter-Cartridge		
		Mean	%CV	S/B	Mean	%CV	S/B
750	577	236843	12	613.6	281727	9	587.2
500	388	162636	18	421.3	203930	21	425.1
400	313	137468	11	356.1	133076	23	277.4
300	238	74534	19	193.1	106839	14	222.7
200	156	47486	24	123.0	58933	30	122.8
100	79	17064	21	44.2	24734	4	51.6
50	39	5892	20	15.3	9116	18	19.0
10	7	882	15	2.3	1363	9	2.8
0	0	386	17		480	17	

[Transferrin] In sample mg/dL	[Transferrin] SIEMENS ADVIA mg/dL	Starting Block Signal (RLU) Inter-Cartridge			Sea Block Signal (RLU) Inter-Cartridge		
		Mean	%CV	S/B	Mean	%CV	S/B
750	577	173128	19	335.1	235474	8	354.6
500	388	147359	14	285.2	214467	12	323.0
400	313	97783	16	189.3	135079	23	203.4
300	238	87934	16	170.2	111812	22	168.4
200	156	42901	23	83.0	57341	26	86.4
100	79	15346	23	29.7	23810	15	35.9
50	39	6306	19	12.2	8002	28	12.1
10	7	1279	10	2.5	1506	12	2.3
0	0	517	28		664	16	

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Figure 6: TRF Effect of Coating Buffers Dose Responses



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2.07 Theranos TRF Standard Curve to Determine ULOQ and LLOQ

The final pair capture # 2 and detector # 13 was used for TRF assay in the Theranos system. Using the assay conditions described below, the TRF assay showed a good detection range from ULOQ at 388 mg/dL and LLOQ at 7 mg/dL. Levels at 577 mg/dL and 4 mg/dL served as the anchor points at the top and bottom of the curve:

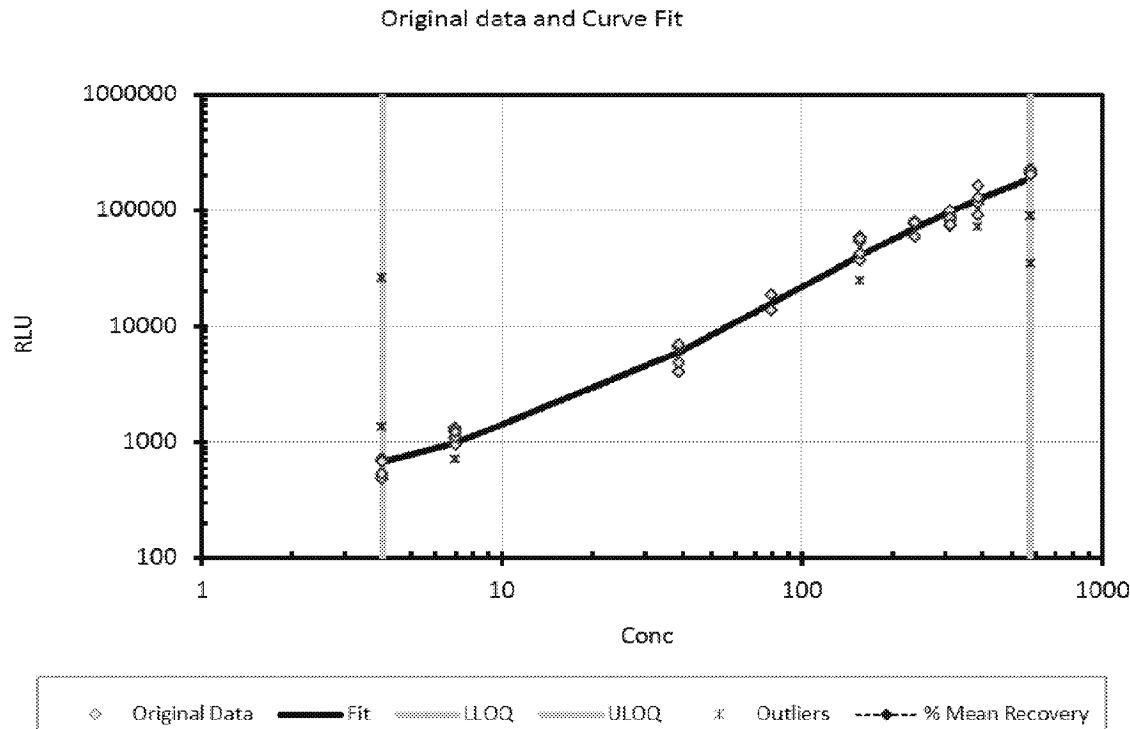
Capture antibody (capture # 2) at 10 ug/ml in Blocking Buffer
Detector antibody at 100 ng/ml (detector # 13) in Theranos in-house AP-Stabilizer with Mg⁺⁺ and Zn⁺⁺ Calibrator set from Cell Sciences prepared in assay buffer
Sample dilution 1:5000x

Table 16: TRF Standard Curve in Assay Buffer

[Transferrin] In sample mg/dL	[Transferrin] SIEMENS ADVIA mg/dL		TRF Signal (RLU)			[TRF back-calculated], mg/dL		% Recovery SIEMENS ADVIA
			Inter Mean	Inter %CV	S/B	Inter Mean	Inter %CV	
750	577		216742	5	829	669	5	116
500	388	*	114654	27	439	359	25	92
400	313		84145	11	322	274	9	88
300	238		73960	13	283	247	11	104
200	156		45481	29	174	169	23	108
100	79		16273	20	62	80	14	102
50	39		5560	25	21	37	20	94
10	7	**	1088	21	4	8	27	113
5	4		605	19	2	3	37	80
0	0		261	17	1	0	N/A	N/A

* This is TRF ULOQ at 388 mg/dL

** This is TRF LLOQ at 7 mg/dL

Figure 7: TRF Standard Curve in Assay Buffer


$$\text{conc} = 84.521 * (((6.071 - 2.410) / (\log_{10}(\text{RLU}) - 2.410)) - 1) ^ {(1 / -0.668)}$$

2.08 Effect of Anticoagulants

To determine the effect of different anticoagulants, 10 matched EDTA plasma and Lithium-heparin plasma samples were tested, and the back-calculated concentration of each sample was determined. Based on the results, it was found that there was no significant difference between EDTA plasma or Lithium-heparin plasma.

Table 17: EDTA vs. Lithium-Heparin Plasma Results

Matrix	Sample #	Signal (RLU)		[TRF back-calculated], mg/dL		SIEMENS ADVIA mg/mL	% Difference from EDTA plasma
		Inter Mean	Inter %CV	Inter Mean	Inter %CV		
EDTA Plasmas	1	94726	13	203	11	244	2
	2	93694	24	202	21	Nd	-16
	3	76948	28	170	24	264	-34
	4	69600	30	156	25	228	-24
	5	108738	20	231	19	275	0
	6	77684	27	171	23	Nd	-24
	7	77094	15	Nd	13	208	Nd
	8	102792	16	219	14	277	23
	9	65126	22	148	18	216	-21
	10	80305	19	176	16	273	-15

	Sample #	Signal (RLU)		[TRF back-calculated], mg/dL		SIEMENS ADVIA mg/mL
		Inter Mean	Inter %CV	Inter Mean	Inter %CV	
Lithium-Heparin Plasmas	1	92528	22	199	19	245
	2	113649	12	241	12	264
	3	120385	24	257	24	259
	4	95334	26	205	23	Nd
	5	118043	16	231	9	265
	6	106476	15	226	14	285
	7	49423	33	Nd	26	198
	8	81148	25	178	21	281
	9	85372	27	186	24	205
	10	96831	20	208	18	268

Nd = Not determined

Additionally, 5 matched serum, EDTA plasma, and Li-hep plasma were tested and it was found that there was no significant difference between the aforementioned samples. These samples were not tested in the reference assay, but the TRF concentrations fell within the reference range of serum TRF in normal population.

Table 18: Additional 5 Matched Serum, EDTA Plasma, and Lithium-hep Plasma Results

Matrix	Sample #	Signal (RLU)		[TRF back-calculated], mg/dL	
		Inter Mean	Inter %CV	Inter Mean	Inter %CV
		1	22	250	22
Serum	2	82317	33	180	28
	3	84256	18	183	16
	4	94928	23	204	21
	5	101292	29	217	26

	Sample #	Signal (RLU)		[TRF back-calculated], mg/dL	
		Inter Mean	Inter %CV	Inter Mean	Inter %CV
		1	9	170	32
EDTA Plasma	2	90895	11	196	10
	3	70543	24	158	19
	4	99056	35	213	32
	5	92437	24	200	22

Lithium-heparin Plasma	Sample #	Signal (RLU)		[TRF back-calculated], mg/dL	
		Inter Mean	Inter %CV	Inter Mean	Inter %CV
		1	22	233	21
Lithium-heparin Plasma	2	90915	9	196	8
	3	78422	14	172	12
	4	103341	11	220	10
	5	92963	31	201	27

2.09 Effect of Interfering Matrices

Hemolyzed, icteric, and lipemic serum samples were obtained from a commercial source, and the TRF concentration for each sample was determined and compared to the reference assay (Siemens Advia 1800). Since the percent difference from the target was relatively higher (greater than 30%), than the desired acceptable range of 20 to 25%, thus it is not recommended to use hemolyzed, icteric, or lipemic samples in the Theranos TRF System 3.0.

Table 19: Effect of Interfering Matrices Results

Theranos TRF System 3.0						
Hemolyzed	Sample ID	RLU	[TRF back-calculated], mg/dL	Siemens ADVIA	% Diff from Target	
		Inter Mean	Inter %CV	Inter Mean	mg/dL	Siemens ADVIA
	1	59391	13	137	10	182
	2	113075	24	241	24	369
	3	72132	16	161	13	290
	4	43867	19	108	14	208
Icteric	5	43060	37	106	28	212
	Sample ID	RLU	[TRF back-calculated], mg/dL	Siemens ADVIA	% Diff from Target	
		Inter Mean	Inter %CV	Inter Mean	mg/dL	Siemens ADVIA
	1	73708	11	164	10	240
	2	66558	13	150	10	194
	3	94917	19	204	17	257
Lipemic	4	66047	21	150	17	229
	5	113917	18	242	17	368
	Sample ID	RLU	[TRF back-calculated], mg/dL	Siemens ADVIA	% Diff from Target	
		Inter Mean	Inter %CV	Inter Mean	mg/dL	Siemens ADVIA
	1	92756	17	200	15	222
	2	59181	10	137	8	272
Lipemic	3	65087	27	148	22	219
	4	84079	18	183	16	253
	5	62851	21	144	17	252

2.10 Effect of Rheumatoid Factor Positive (Rf+) Serum Samples

Rheumatoid Factor Positive (Rf+) serum samples were obtained from a commercial source and the TRF concentration for each sample was determined. However, these samples were not tested in the reference assay, but the TRF concentrations fell within the reference range of serum TRF in normal population.

Table 20: Effect of Rf+ Serum Samples Results

Sample #	Rf+ Samples			
	Signal (RLU)		[TRF back-calculated], mg/dL	
	Inter Mean	Inter %CV	Inter Mean	Inter %CV
1	83112	20	182	18
2	74201	15	165	12
3	87415	19	189	16
4	78322	21	172	18
5	111815	13	237	12
6	87419	15	189	13
7	87742	12	190	11
8	99164	16	212	14

2.11 Effect of HAMA Serum Samples

HAMA serum samples were obtained from a commercial source and the TRF concentration for each sample was determined (Table 21), and again, these samples were not tested in the reference assay, but the TRF concentrations fell within the reference range of serum TRF in normal population.

Table 21: Effect of HAMA Serum Samples Results

Sample #	HAMA Samples		[TRF back-calculated], mg/dL	
	Signal (RLU)		Inter Mean	Inter %CV
	Inter Mean	Inter %CV		
1	47813	18	116	14
2	78815	20	173	17
3	109253	12	232	11
4	53768	21	127	17
5	123623	21	263	22

2.12 Final Clinical Correlation against Clinical Analyzer such as the Siemens Advia 1800

Twenty-six clinical samples were run on the Theranos TRF System 3.0 with the above final assay conditions. The Percent Different from the reference method (Siemens Advia 1800) was determined, and 19 samples met the acceptable range of 20 to 25%; 3 samples were between 25.1% to 28.1%; and 4 samples greater than 28.2%.

As a final test to validate the Theranos TRF System 3.0, an IRMM "QC Control" was used and the back-calculated concentration determined to be 195 mg/dL vs. the reported concentration of 236 mg/dL, and the percent different from the reference method was -17.2%.

Table 22: Final Clinical Correlation of Theranos TRF System 3.0 vs. Siemens Advia 1800

Sample #	Sample ID	SIEMENS_ADVIA mg/dL	Theranos TRF System 3.0				
			Signal (RLU)		[TRF, back-calculated], mg/dL	% Diff from Target	
			Inter-Cartridge	Inter-Cartridge	SIEMENS_ADVIA		
Mean	%CV	Mean	%CV	mg/dL			
1	AD08	293	85703	21	279	18	-4.9
2	AD10	239	58676	14	205	11	-14.0
3	AD18	312	88990	19	287	16	-7.9
4	P7	201	37947	20	148	15	-26.5
5	P11	414	114683	11	358	10	-13.6
6	P12	333	93325	25	299	21	-10.1
7	L3	215	46044	20	171	15	-20.7
8	L4	290	64780	36	222	28	-23.6
9	6-BRH539339	259	60136	7	210	6	-19.1
10	10-BRH539334	314	86289	8	280	6	-10.8
11	P1	283	71893	9	241	7	-14.8
12	P3	232	56759	32	212	21	-8.6
13	BRH460987	345	100216	21	318	18	-7.8
14	BRH460980	269	56909	24	200	18	-25.5
15	PD6	248	52493	27	188	21	-24.1
16	PD7	115	21820	28	99	20	-14.2
17	PD8	276	82295	21	269	18	-2.4
18	PD9	229	49823	24	163	30	-28.8
19	PD10	147	26214	25	113	17	-23.3
N/A	IRMM	236	54947	15	195	12	-17.2
20	AD03	312	64128	27	234	17	-25.1
21	AD05	247	49099	33	187	23	-24.3
22	AD07	320	77237	26	256	21	-20.1
23	AD11	208	40937	35	156	26	-25.1
24	7-BRH539338	299	62332	41	215	32	-28.1
25	8-BRH539336	300	39212	20	151	15	-49.5
26	9-BRH539340	239	72963	16	244	13	2.1
27	PD11	284	42540	39	160	29	-43.6



2.13 Stability Studies: on-going

The following are the proposed guidelines for the stability studies:

I) for the capture: the replicates are stored at 2 conditions: 4°C and room temperature (RT) and are monitored over the course of a 3-month period.

II) for the detector: the working concentration is prepared on day 1 and tested over the course of a 3-month period against the control detector.