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Cardiac Troponin T (cTNT) 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"]

This assay is designed to detect cardiac Troponin T (cTNT) in human plasma, serum and whole blood. The assay has a reportable range of 25 -0.0125 ng/mL. Due to the lack of availability of an internationally accepted reference standard for this assay, the Theranos cTNT is calibrated on the nominal concentrations of a commercially available native cTNT analyte.

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

The only available commercial electrochemiluminescent immunoassay for cTNT is the Roche Elecsys 2010 clinical analyzer platform. All clinical samples and calibrator material were tested and verified on the Roche cTNT assay by shipping samples out to a central testing facility.
following commercial ELISA kits have been used in house as predicate methods:

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

A biotin labeled mouse anti-cTNT antibody coated on an avidin surface serves as the capture surface for this sandwich ELISA. The sample (whole blood, plasma and serum) is diluted with the diluent and is followed by mixing with the alkaline phosphatase labeled mouse anti-cTNT antibody. The mixture is then incubated on the capture surface for 5 minutes. Following this the surface is washed and the substrate is incubated on the surface for 5 minutes. The resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Human Cardiac Troponin T	Scripps Laboratories	T1514
Mouse Anti Cardiac Troponin T Antibody (Capture), Clone # 1c11	Fitzgerald	10R-T127d
Mouse Anti Cardiac Troponin T Antibody (Detection), Clone #M8020207	Fitzgerald	10-T85D
Phospho Glo Substrate	KPL	55-60-04
Starting Block (TBS) Blocking Buffer	Pierce	37542
Blocking Buffer (3% BSA in TBS, 0.05% Sodium Azide)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G
Carbonate-bicarbonate buffer	Sigma	C3041
Sodium Citrate(trisodium citrate dihydrate)	Sigma	S1804-500G
Citric Acid	Sigma	C1909-500G
Urea	Sigma	U5378-100g
β-mercaptoethanol	Sigma	M7522-100ML
Troponin I depleted serum	Sunny Lab	SF184-2

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

1.2 Antibody Screening

The Antibody screening on the MTP had to be performed in 2 rounds. In Round 1 20 antibodies were tested in all possible combinations using the native CTNT analyte. Due to the lack of sensitivity seen at 0.1 ng/mL the screening had to be expanded to include 20 more antibodies in the second round. Round 2 screening was performed with three levels of the native cTNT (2, 0.5 and 0 ng/mL) run neat as well as three levels of the commercially available Bio Rad Cardiac Control samples, diluted 1:5 in blocking buffer, for each sandwich combination. Table 2 lists all the antibodies tested. The expanded screen afforded 6 pairs of antibodies that had good signal to background and high over low values for the analyte and commercial controls tested respectively. Table 3 provides a summary of the antibody screen and Table 4 summarizes the data for the best pairs identified.

Table [SEQ Table * ARABIC]: List of antibodies tested for cTNT

Serial No.	Vendor	Cat#	Clone#	Clonality
1	R&D	MAB1874	200805	mouse monoclonal
2	Thermo scientific	MA1-16688	7A9	mouse monoclonal
3	Thermo scientific	MA1-24612	1F11	Mouse monoclonal
4	Thermo scientific	MA1-24614	2F3	mouse monoclonal
5	Thermo scientific	MA1-20879	7E7	Mouse monoclonal
6	Novus biological	NB120-8296	9G6	mouse monoclonal
7	Abcam	ab45932		rabbit polyclonal
8	My Biosource	MBS662002		goat polyclonal
9	My Biosource	MBS462149		rabbit poly
10	Thermo scientific	MA1-24621	7G7	mouse monoclonal
11	Thermo scientific	MA1-24615	2G3	mouse monoclonal
12	Thermo scientific	MA1-24611	1A11	mouse monoclonal
13	Thermo scientific	MA1-16687	1C11	mouse monoclonal
14	Thermo scientific	MA1-24616	1F2	mouse monoclonal
15	Thermo scientific	MA1-24614	3D6	mouse monoclonal
16	Abcam	ab999		mouse monoclonal
17	Abcam	ab89221		mouse monoclonal
18	Abcam	ab998		mouse monoclonal
19	QED Bioscience	24101		mouse monoclonal
20	QED Bioscience	24102		mouse monoclonal
21	Mybiosource	MBS531260	M322512	mouse monoclonal
22	Mybiosource	MBS462147		rabbit polyclonal
23	Novus biological	NB120-10222	7F4	mouse monoclonal
24	US biological	T8665-23Y	9F339	mouse monoclonal
Serial No.	**Vendor**	**Cat#**	**Clone#**	**Clonality**
25	US biological	T8665-23G	9F342	mouse monoclonal
26	US biological	T8665-23B	9L745	mouse monoclonal

27	US biological	T8665-23U	9F146	mouse monoclonal
28	US biological	T8665-23P	9F142	mouse monoclonal
29	US biological	T8665-23T	9F145	mouse monoclonal
30	US biological	T8665-23J	9F137	mouse monoclonal
31	US biological	T8665-23K	9F138	mouse monoclonal
32	US biological	T8665-23M	9F140	mouse monoclonal
33	US biological	T8665-23N	9F141	mouse monoclonal
34	US biological	T8665-22C1	10F567	mouse monoclonal
35	US Biological	T8665-30D	5E846	mouse monoclonal
36	US biological	T8665-30G	5E849	mouse monoclonal
37	US biological	T8665-22B	1.BB.156	mouse monoclonal
38	US biological	T8665-22D	8.F.291	mouse monoclonal
39	US biological	T8665-23C	2Q1102	mouse monoclonal
40	US biological	T8665-30A	5E843	mouse monoclonal

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

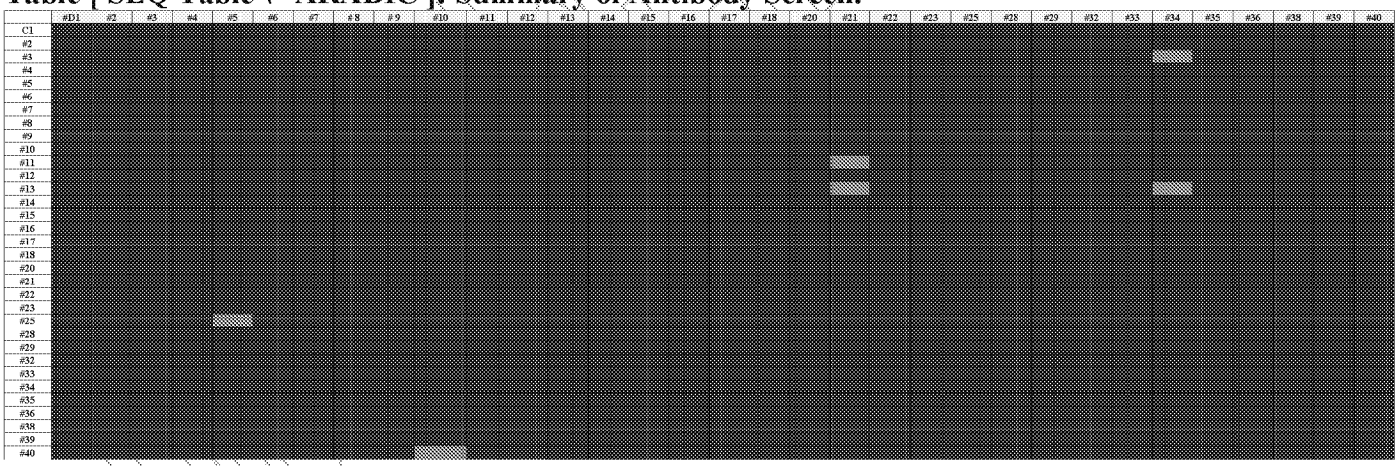


Table [SEQ Table * ARABIC]: Summary of Best Pairs (MTP)

Cab	Dab		[cTNT] pg/mL	Mean RLU	% CV	S/B or High/Low	Std 2/Std 3
40	10	Analyte in buffer	2000	24036	14	53	13
			500	6064	2		
			0	450	36		
		Serum based Cardiac Control	2320	65387	0	31	
			487	8329	1		
			107	2142	4		
11	21	Analyte in buffer	2000	13133	1	44	15
			500	4359	8		
			0	298	5		
		Serum based Cardiac Control	2320	35514	2	29	
			487	4191	1		
			107	1224	10		
13	21	Analyte in buffer	2000	22047	1	80	17
			500	4748	6		
			0	276	13		
		Serum based Cardiac Control	2320	28330	2	40	
			487	2771	13		
			107	710	3		
3	34	Analyte in buffer	2000	10506	9	44	11
			500	2608	10		
			0	239	9		
		Serum based Cardiac Control	2320	20266	7	23	
			487	2337	5		
			107	865	9		
25	5	Analyte in buffer	2000	7222	26	34	10
			500	2098	4		
			0	213	3		
		Serum based Cardiac Control	2320	9910	6	20	
			487	1170	14		
			107	504	3		
13	34	Analyte in buffer	2000	9997	40	40	14
			500	3496	13		
			0	252	25		
		Serum based Cardiac Control	2320	20606	0	25	
			487	2824	0		
			107	809	34		

Important note: The antibody pair C40D10 emerged as a candidate at the end of more experiments following the antibody screen above that included cross reactivity, interference and testing the effect of various matrices (depleted serum, normal serum, normal plasma and whole blood) (data not shown). However, a training set of samples tested with this pair failed to produce any dose response and resulted in a very poor correlation with the result from the Roche cTNT assay run externally for these same samples.

It was decided to abandon this antibody pair and restart screening efforts this time using clinical samples that had been tested on the Roche cTNT assay. Candidate pairs for the next round of screening were chosen based on important epitope information gathered from recent literature. The screening was performed directly on the Theranos system.

1.3 Antibody Screening on Theranos System

This round of screening introduced another mouse anti- CTNT antibody which was designated #41. Along with the control pair, C40D10, 5 additional combinations including Ab#41 were evaluated for the best dose response seen with clinical samples. Table 5 summarizes the data. Ab pair C13D41 emerged as a candidate pair that showed the best modulation between the high and low clinical samples. This pair along with another, C41D10, which showed the next best modulation, was picked for further evaluation.

Table [SEQ Table * ARABIC]: Results of Antibody Screening with Clinical Samples on Theranos System

Pooled clinical samples	C40D10 (Ctrl)		C41D10		C13D10		C13D23		C41D23		C13D41	
[cTNT] ng/mL	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
High (2.68-1.93)	3359	7	6007	5	3396	5	58168	20	277817	2	544931	12
Medium (1.5-1.7)	1979	25	4385	6	1920	6	30967	22	223978	8	252328	7
Low (1.09-1.1)	1833	7	2937	15	1501	14	43062	14	249803	22	196468	6
0 (<0.010)	1820	5	1585	10	1199	18	34076	13	238746	1	40738	12
High/Low	1.8		3.8		2.8		1.7		1.2		13.4	

1.4 Clinical Sample Response Optimization

Table 6 summarizes the results of further testing Ab pairs C13D41 and C41D10 with more clinical samples. Following this C13D41 was picked for further evaluation. This included testing the response to clinical samples with different detection antibody stabilizers as summarized in Table 7. Side by side evaluation of cTNT native calibrator material revealed an offset between the clinical sample and the calibrator material responses. This was seen to be the case if the calibrator concentrations were assigned based on the results of running them on the Roche cTNT assay (Table 8).

Table [SEQ Table * ARABIC]: Clinical sample testing with Ab pairs C13D41 and C41D10.

			C13D41		C41D10	
ProMedDx ID	[cTNT] reported ng/mL	Matrix	Inter-Cartridge Mean	CV%	Inter-Cartridge Mean	CV%
11663212	10.8	Serum	879088	15	1835	9
11663458	8.22	Serum	1389557	5	2194	7
11663155	4.84	Serum	472458	9	1500	10
11663244	3.7	Serum	392500	7	1745	12
11660952	3.43	Li-Heparin Plasma	220939	11	2047	9
11482547	2.99	Li-Heparin Plasma	153119	1	3463	1
11417719	1.55	Li-Heparin Plasma	392450	6	9071	10
2011409	1.02	Li-Heparin Plasma	858458	9	14369	8
1575954	0.76	K3EDTA Plasma	171450	10	6030	9
1575947	0.27	K3EDTA Plasma	117131	16	1794	22
1578785	0.05	K3EDTA Plasma	71185	25	1647	22
Stanford donor	0	Li Heparin plasma	20088	22	1242	15
Stanford donor	0	Li Heparin plasma	20244	5	1115	21
1575956	0	K3EDTA Plasma	57557	5	1496	40
		H/L	69			

Table [SEQ Table * ARABIC]: Further optimization of Clinical Sample response: Ab pair C13D41

			5 ng/mL D41 in Biostab			100 ng/mL D41 in StabilZyme		
ProMedDx ID	cTNT reported ng/mL PPD/ProMedDx	Matrix	Inter-Cartridge Mean	CV%	S/B	Inter-Cartridge Mean	CV%	S/B
11663458	8.22	Serum	364411	7	100	984849	15	465
11663155	4.84	Serum	120087	8	33	249001	16	118
1575954	0.76	K3EDTA Plasma	31065	5	9	35119	10	17
1575947	0.27	K3EDTA	29936	12	8	27854	26	13
1578785	0.05	K3EDTA	16831	10	5	5288	15	2
Stanford donor	0	Li Heparin plasma	3640	8	1	2116	35	1

Table [SEQ Table * ARABIC]: cTNT Standard Curve

		5 ng/mL D41 in Biostab			100 ng/mL D41 in StabilZyme		
Nominal	PPD reported	Inter-Cartridge RLU		S/B	Inter-Cartridge RLU		S/B
ng/ml	ng/mL	Mean	CV%		Mean	CV%	
8	2.83	420961	12	87	669289	11	161
4	1.32	152888	13	32	162124	8	39
2	0.6	95456	13	20	85833	9	21
0.5	0.16	31208	8	6	24984	12	6
0.1	0.02	10226	11	2	5940	25	1
0	0	4817	18	1	4164	31	1
	(OORL)						

1.5 Assay Format Optimization

In order to elicit the maximum dose response out of the clinicals it was decided to investigate different sandwich ELISA assay formats to test the effect on clinical sample dose response. The control format was a 2 step format where the sample was diluted and incubated on the surface for 10 minutes followed by 10 minute incubation with the Dab followed by wash and incubation for 10 minutes with the substrate. As an alternate 2 homogeneous assay formats were tested (i) where Avidin was on the surface and all the sample, Cab and Dab were in solution and (ii) where the Capture Ab was on the surface and the sample was mixed with Dab . Out of the three formats tested homogeneous format (ii) afforded the best dose response amongst the clinicals tested (Table 9).

Table [SEQ Table * ARABIC]: Effect of Assay Formats on the Clinical Sample Dose Response

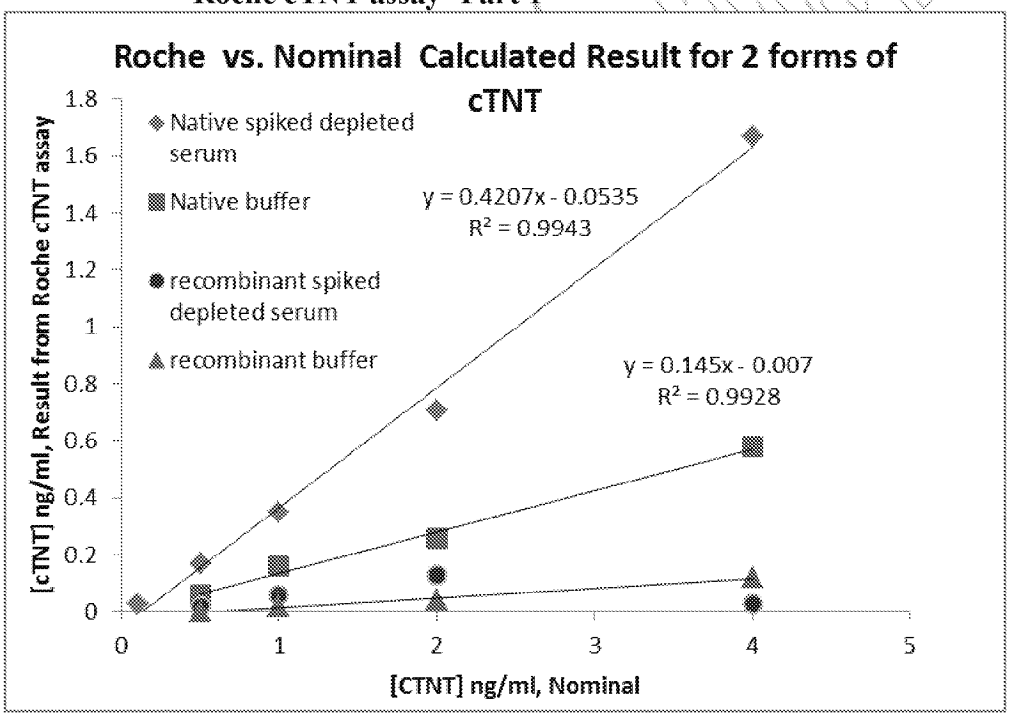
CLINICAL SAMPLES		2 step format Capture Ab on surface			Homogeneous format (sample, Cab and Dab in solutio Avidin surface			Homogeneous format (sample and Dab in solution) Capture Ab on surface		
ProMedDx ID	[cTNT] reported ng/mL	Inter-Cartridge RLU Mean	CV%	S/B	Inter-Cartridge RLU Mean	CV%	S/B	Inter-Cartridge RLU Mean	CV%	S/B
11663458	8.22	364411	7	100	8783	21	34	49242	10	132
11663155	4.84	120087	8	33	1544	7	6	10058	1	27
1575954	0.76	31065	5	9	321	1	1	2025	14	5
1578785	0.05	16831	10	5	259	12	1	1060	18	3

cTNT native Standard curve		2 step format Capture Ab on surface			Homogeneous format (sample, Cab and Dab in solutio Avidin surface			Homogeneous format (sample and Dab in solution) Capture Ab on surface		
[cTNT] nominal ng/mL	[cTNT] reported ng/mL	Inter-Cartridge RLU Mean	CV%	S/B	Inter-Cartridge RLU Mean	CV%	S/B	Inter-Cartridge RLU Mean	CV%	S/B
8	2.83	420961	12	87	8902	24	24	31180	21	83
4	1.32	152888	13	32	3168	8	8	13701	8	37
1	0.31	31208	8	6	1129	17	3	3143	8	8
0	0	4817	18	1	376	13	1	374	22	1

1.6 Calibrator Comparison Part 1

Troponin T is available in three forms commercially: (i) native (purified from human cardiac muscle), (ii) recombinant (overexpressed in *E. coli*) and (iii) as part of the troponin ternary complex (I-T-C) with troponin I and troponin C. The native and recombinant forms were spiked into commercially available troponin I depleted serum at levels spanning the range of the assay and tested on the Roche cTNT assay at an external facility. There was a distinct difference between the recognition of the two forms by the Roche assay. Further differences were seen between the spiked depleted serum and buffer based calibrators. The native spiked depleted serum calibrators had a modest response on the Roche assay (slope of 0.42) compared with the nominal concentrations (Figure 1).

Figure [SEQ Figure * ARABIC]: Performance of Theranos cTNT calibrators on the Roche cTNT assay- Part 1



1.7 Effect of Calibrator Diluent

Reports on the third generation Roche cTNT assay development indicated that the cTNT calibrator material was prepared by spiking in bovine CTNT into pH 5.6 citrate-buffered human serum. It was also reported that the calibrator material was switched from the bovine to the recombinant version in the third generation Roche. Based on this report preliminary tests were performed in which all three forms of the cTNT available in house were first formulated in (i) 3% BSA/0.05% NaN_3 , pH 8.0 and (ii) 100 mM citrate buffer, pH 5.6 followed by spiking into Troponin I depleted serum. The hypothesis being that the analyte was at a pH incompatible formulation and was being degraded. The results revealed that the lowering of the pH in the calibrator formulation helps in linearizing the dose response (Figures 2-3). Calibrators made in this manner, for all three forms of cTNT, were tested on the Roche assay at an external facility.

Figure [SEQ Figure * ARABIC]: Dose response comparison of CTNT spiked depleted serum calibrators with citrate formulation.

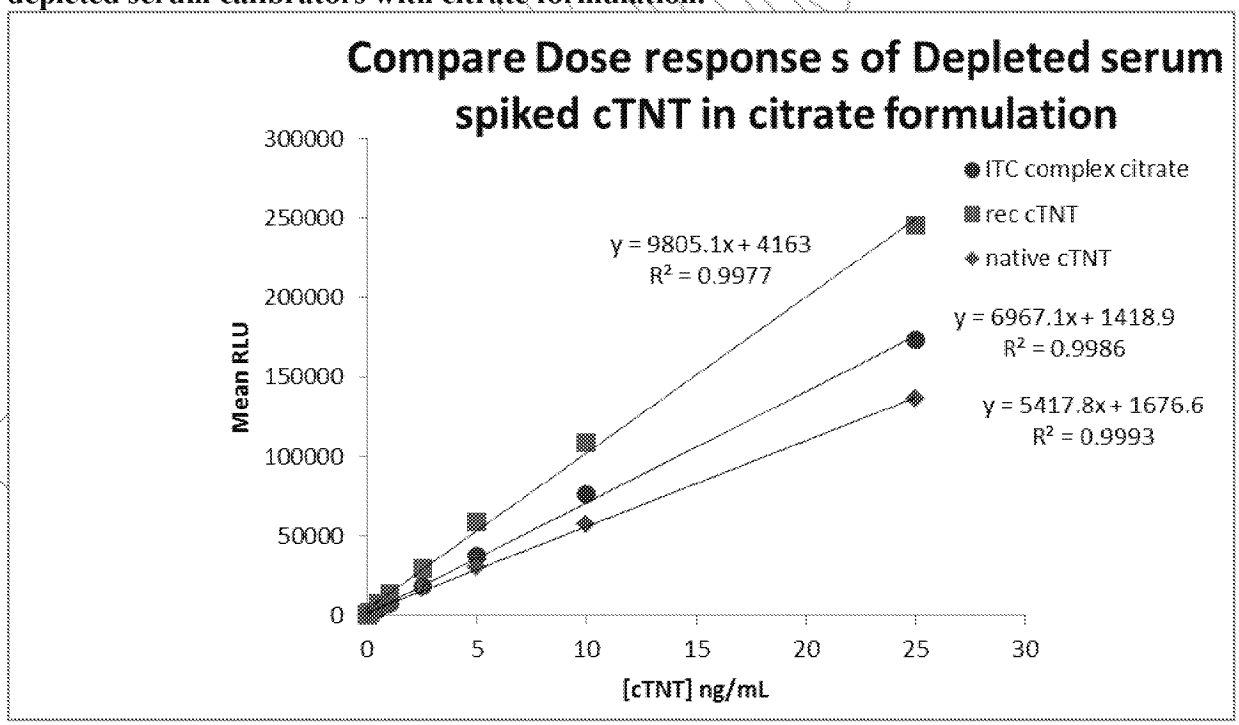
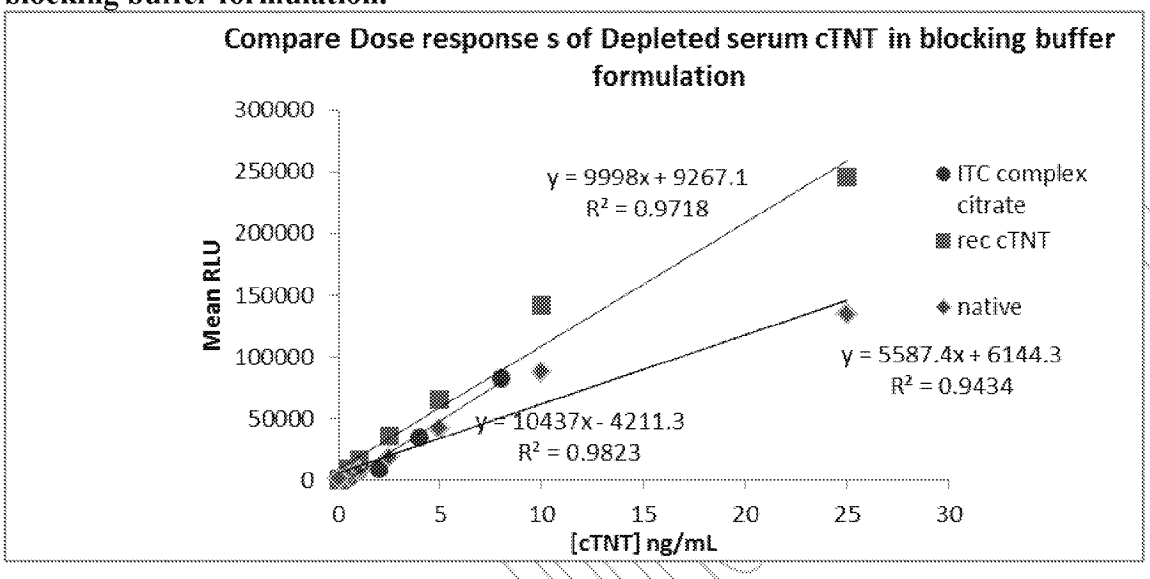


Figure [SEQ Figure * ARABIC]: Dose response comparison of CTNT spiked depleted serum calibrators with blocking buffer formulation.



1.8 Effect of low pH citrate buffer as assay diluent

The low pH buffer formulation was extended to the assay diluent in order to see if this was important in the recognition and binding of the cTNT in the clinical samples to the antibodies. The control assay diluent was blocking buffer which was at a higher pH 8.0. Clinical samples and the three calibrator materials were tested side by side with both the diluents. The calibration curve results are summarized in tables 10 and 11 and depicted in Figures 4 and 5. The low pH phosphate – citrate buffer, pH 6.4 diluent causes saturation in the dose response of the calibrators. Tables 12 and 13 summarize the data for the clinicals obtained using the two diluents. Figures 6-11 depict the clinical correlation data. Although not detrimental to the overall clinical correlation the low pH diluent affected the slope of the correlation curve which implied that recoveries were less than optimal (recoveries need to be within 20% of the reference value). It was decided not to pursue the low pH buffer formulation for the assay diluent.

Table [SEQ Table * ARABIC]: Standard curves: Phosphate Citrate buffer pH 6.4 as diluent

Nominal ng/mL	Rec CTNT	Native	rec ITC complex
25	264235	150711	198226
10	140531	74372	119291
5	94668	37490	60544
2.5	61471	25503	22172
1	26345	9910	10427
0.5	10411	6544	4731
0.1	2967	2577	1421
0.025	1558	1774	921
0	807	807	807
S/B	327	187	246
Std 8/Std 9	1.9	2.2	1.1

Table [SEQ Table * ARABIC]: Standard Curves: Blocking buffer as diluent

Nominal ng/mL	Rec CTNT	Native	rec ITC complex
25	245665	136210	173440
10	108293	57040	76249
5	59124	30320	37301
2.5	29225	16781	18211
1	14095	6903	7617
0.5	7102	3497	3986
0.1	1789	1509	1579
0.025	1443	971	1328
0	417	519	503
S/B	589	262	345
Std 8/Std 9	3.5	1.9	2.6

Figure [SEQ Figure * ARABIC]: Standard curves: Phosphate Citrate buffer pH 6.4 as diluent

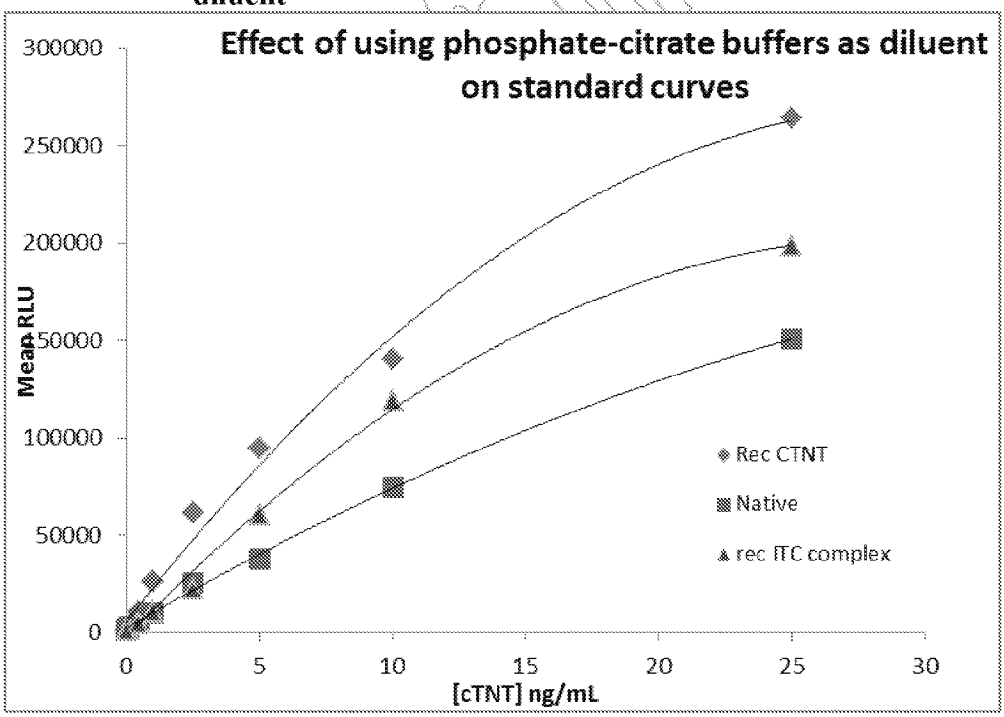


Figure [SEQ Figure * ARABIC]: **Standard Curves: blocking buffer as diluent**

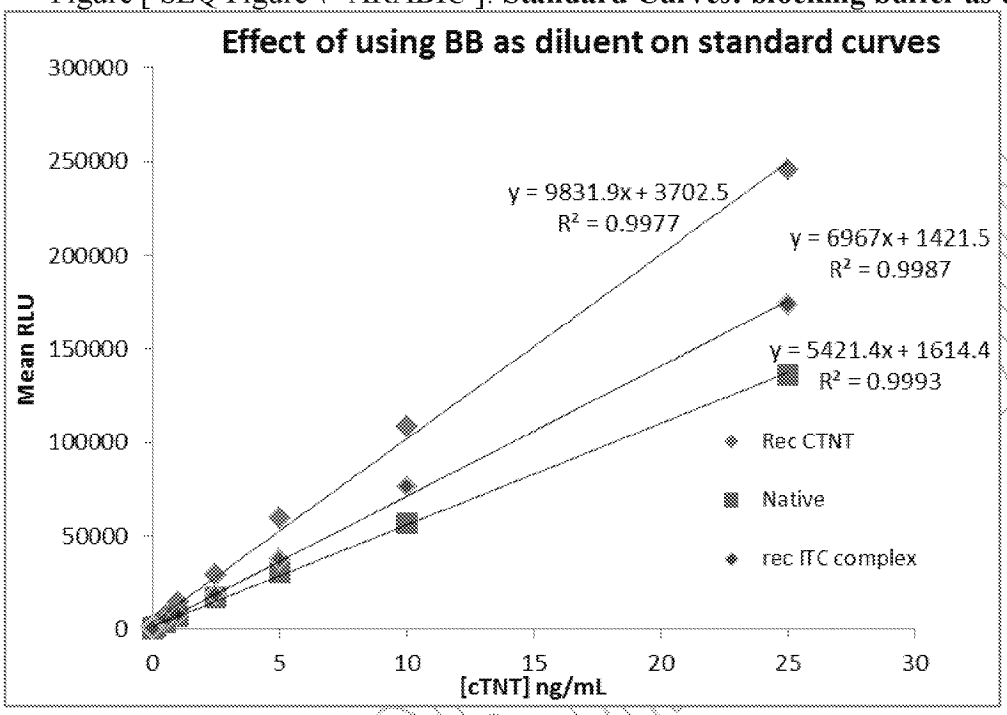


Table [SEQ Table * ARABIC]: Clinical Samples: Effect of low pH buffer diluent

CTNT reported	Inter-Cartridge		rec CTNT calibration			native CTNT calibration			rec ITC complex calibration		
ng/mL PPD/ProMedDx	Mean	CV%	Mean	CV%	% Recovery	Mean	CV%	% Recovery	Mean	CV%	% Recovery
10.8	50621	11	2.20	12	20	6.08	12	56	4.11	10	38
8.22	101622	21	5.27	31	64	14.58	31	177	8.86	28	108
7.47	71046	13	3.25	16	44	9.01	16	121	5.76	14	77
4.84	22774	9	1.00	8	21	2.62	9	54	2.13	7	44
3.79	3114	11	0.10	18	3	0.13	26	3	0.31	16	8
3.43	52237	12	2.28	13	67	6.29	13	183	4.23	11	123
2.99	7467	6	0.33	7	11	0.66	9	22	0.86	6	29
2.8	20040	7	0.89	7	32	2.29	8	82	1.93	5	69
2.05	37286	15	1.61	15	78	4.38	16	214	3.14	13	153
1.7	25358	16	1.11	15	65	2.93	17	172	2.31	12	136
1.64	20449	6	0.91	5	55	2.34	6	142	1.96	4	120
1.4	9564	9	0.43	9	31	0.94	12	67	1.08	8	77
1.02	20189	1	0.89	2	87	2.27	3	223	1.93	2	189
0.27	2537	7	0.07	12	26	0.08	17	29	0.23	11	85
0.05	1963	17	0.04	34	87	0.04	47	80	0.15	30	301

Table [SEQ Table * ARABIC]: Clinical Samples: Effect of Blocking buffer as diluent

CTNT reported	Inter-Cartridge		Backcalculation (ng/ml)								
ng/mL	Mean	CV%	rec CTNT			native CTNT			rec ITC complex		
PPD/ProMedDx	RLU		Mean	CV%	% Recovery	Mean	CV%	% Recovery	Mean	CV%	% Recovery
10.8	41549	10	3.40	10	32	6.41	10	59	5.23	8	48
8.22	49242	10	4.08	10	50	7.58	11	92	6.04	9	73
7.47	86808	4	7.23	4	97	14.29	5	191	10.51	4	141
4.84	10058	1	0.67	26	14	1.71	0	35	1.57	1	32
3.79	5575	14	0.38	18	10	0.88	18	23	0.78	19	21
3.56	14800	12	1.27	14	36	2.50	11	70	2.27	11	64
3.43	37742	20	3.10	19	90	5.85	19	171	4.83	16	141
3.23	13342	9	1.14	13	35	2.27	9	70	2.07	8	64
2.8	13841	11	1.13	12	40	2.35	11	84	2.14	10	76
2.61	3719	1	0.21	18	8	0.50	2	19	0.42	2	16
2.05	19499	15	1.61	15	79	3.21		157	2.86	12	140
1.8	10239	19	0.80	24	44	1.75	19	97	1.60	19	89
1.64	9899	5	0.78	6	48	1.69	5	103	1.56	5	95
1.4	5675	6	0.39	9	28	0.90	8	64	0.80	9	57
1.02	1788	17	0.04	75	4	0.14	30	13	0.10	34	9
0.76	2025	14	0.07	20	9	0.17	28	22	0.12	32	16
0.27	3539	16	0.20	24	73	0.46	24	171	0.38	27	142
0.05	1060	18	0.02	43	42	0.04	49	74	0.02	57	40

Figure [SEQ Figure * ARABIC]: Clinical correlation: rec cTNT low pH buffer diluent

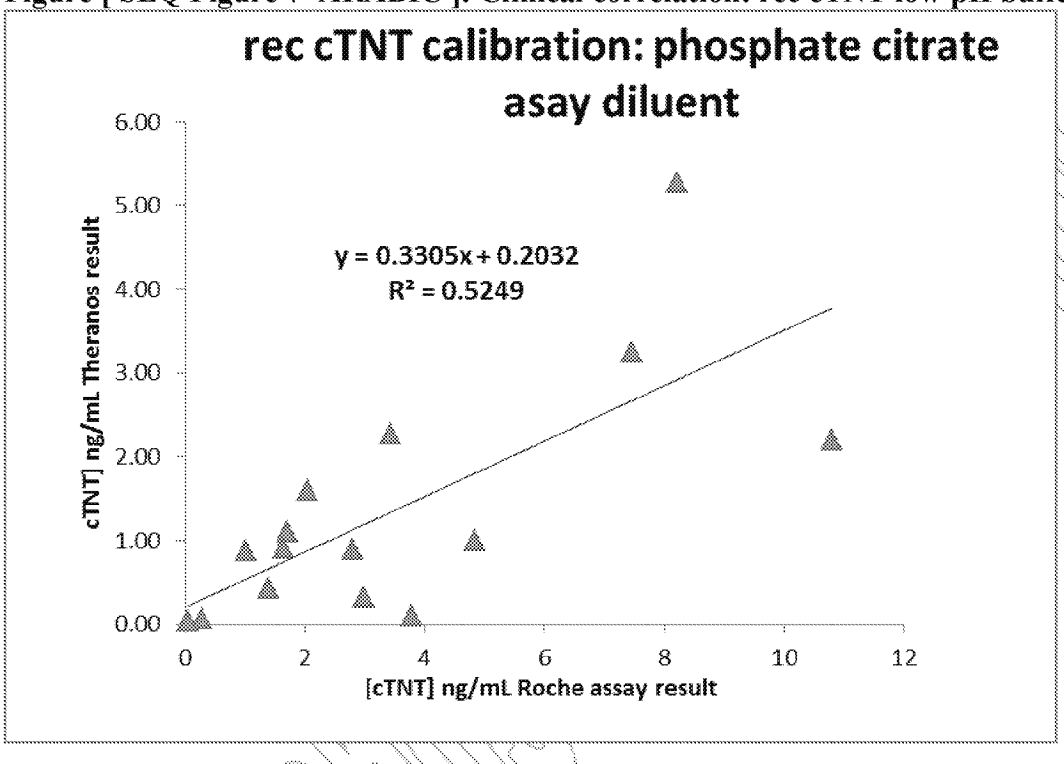


Figure [SEQ Figure * ARABIC]: Clinical correlation: native cTNT low pH buffer diluent

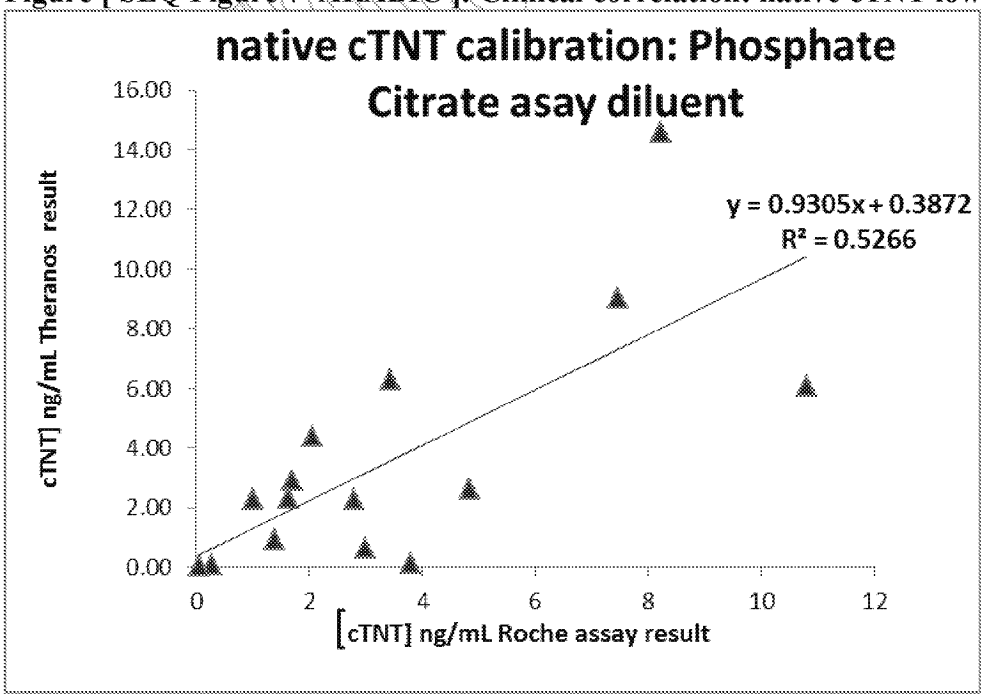


Figure [SEQ Figure * ARABIC]: Clinical correlation: Troponin complex low pH buffer diluent

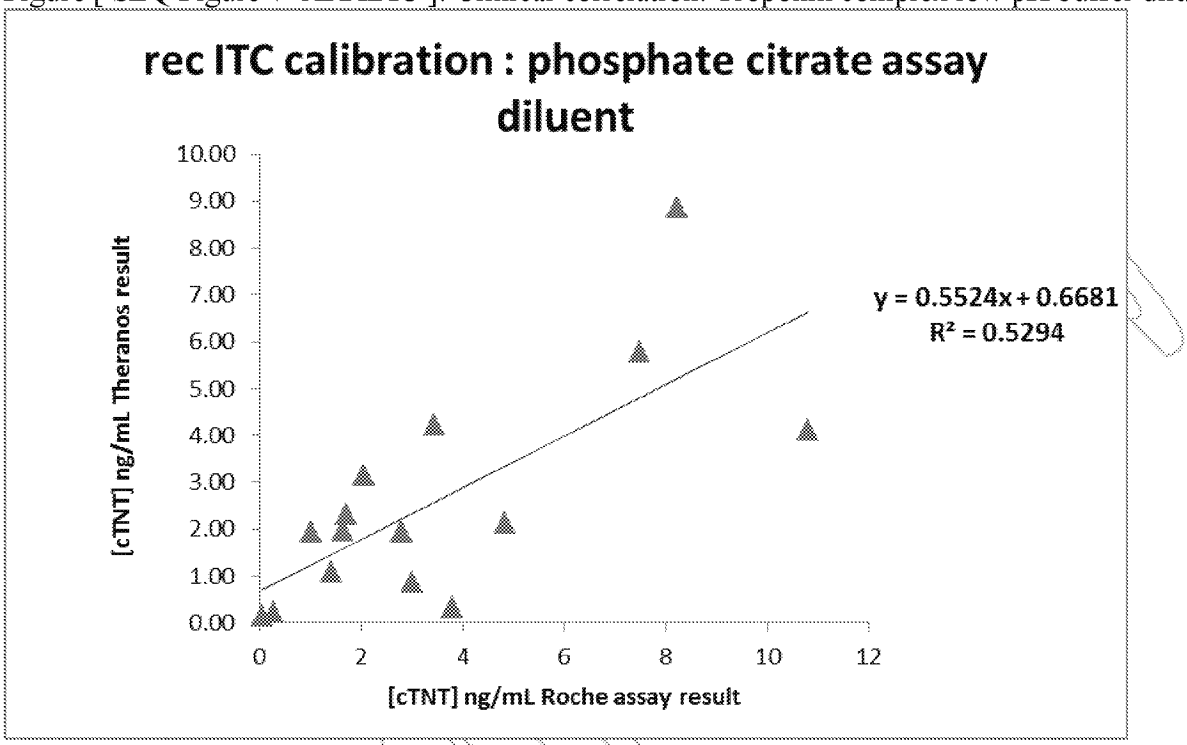


Figure [SEQ Figure * ARABIC]: Clinical correlation: rec cTNT blocking buffer diluent

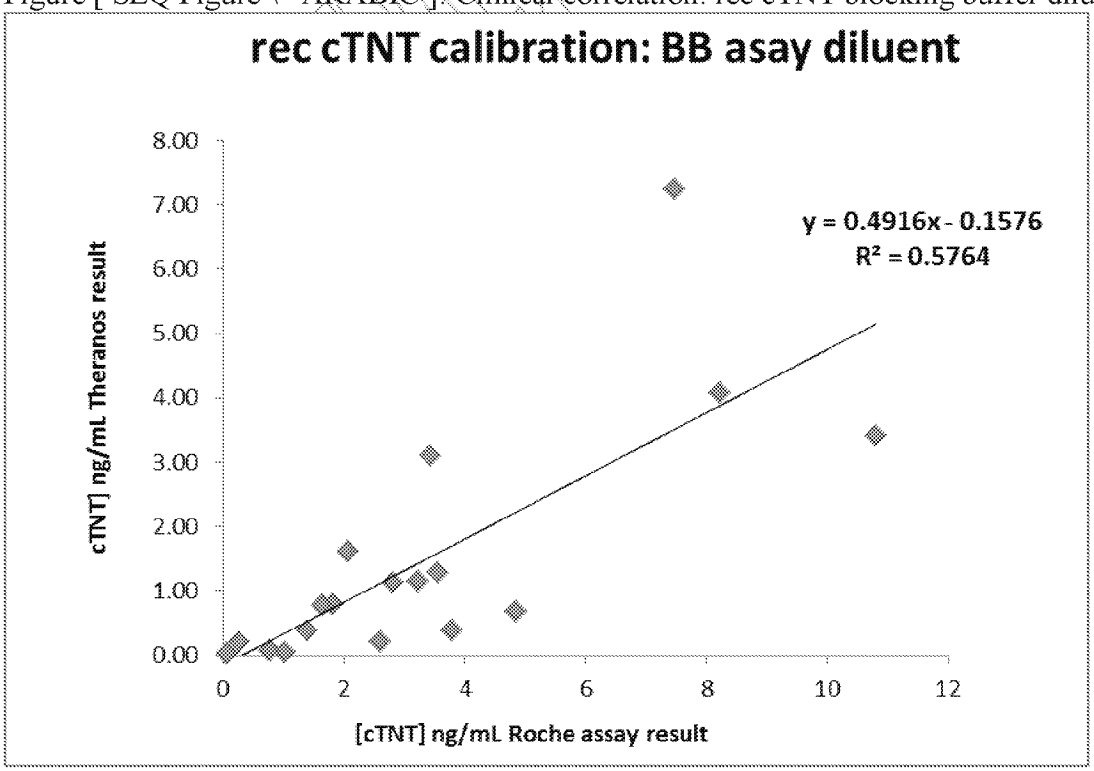


Figure [SEQ Figure * ARABIC]: Clinical correlation: native cTNT blocking buffer diluent

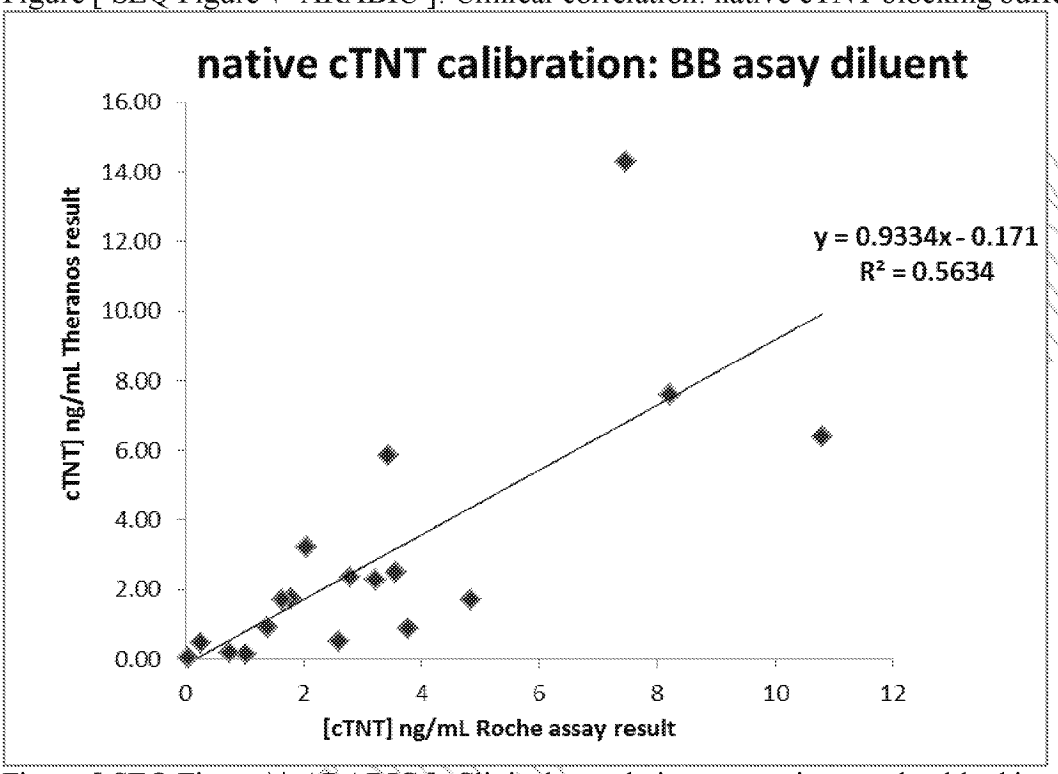
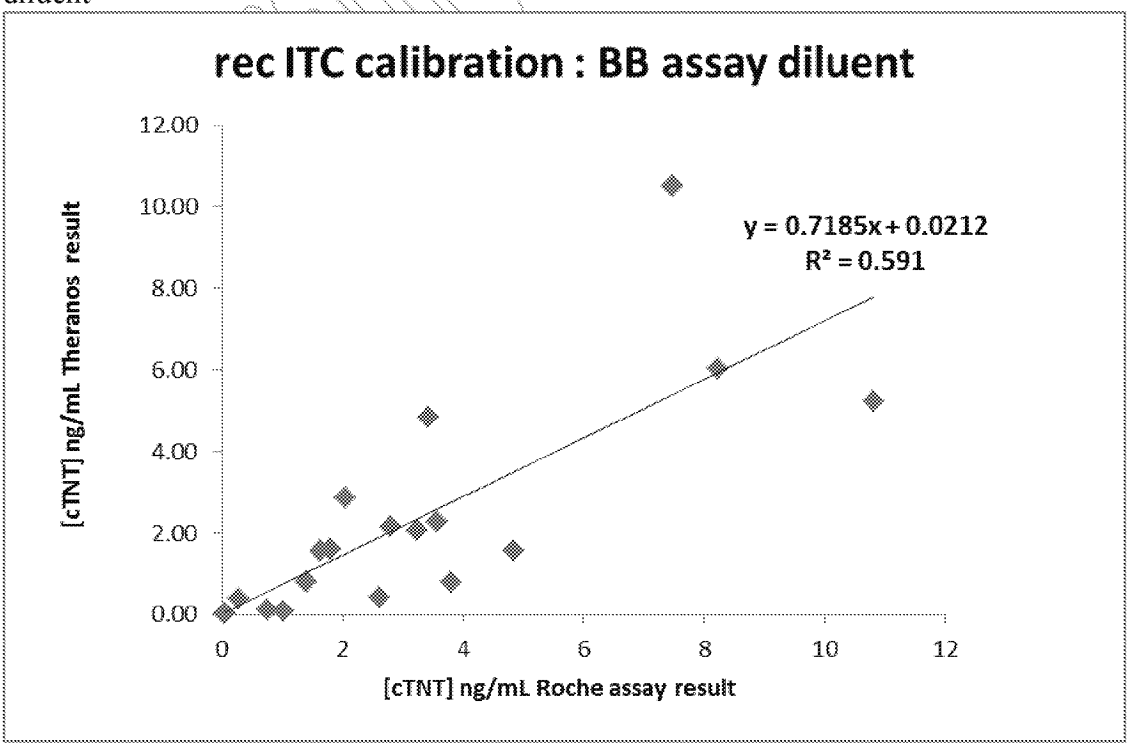


Figure [SEQ Figure * ARABIC]: Clinical correlation: troponin complex blocking buffer diluent



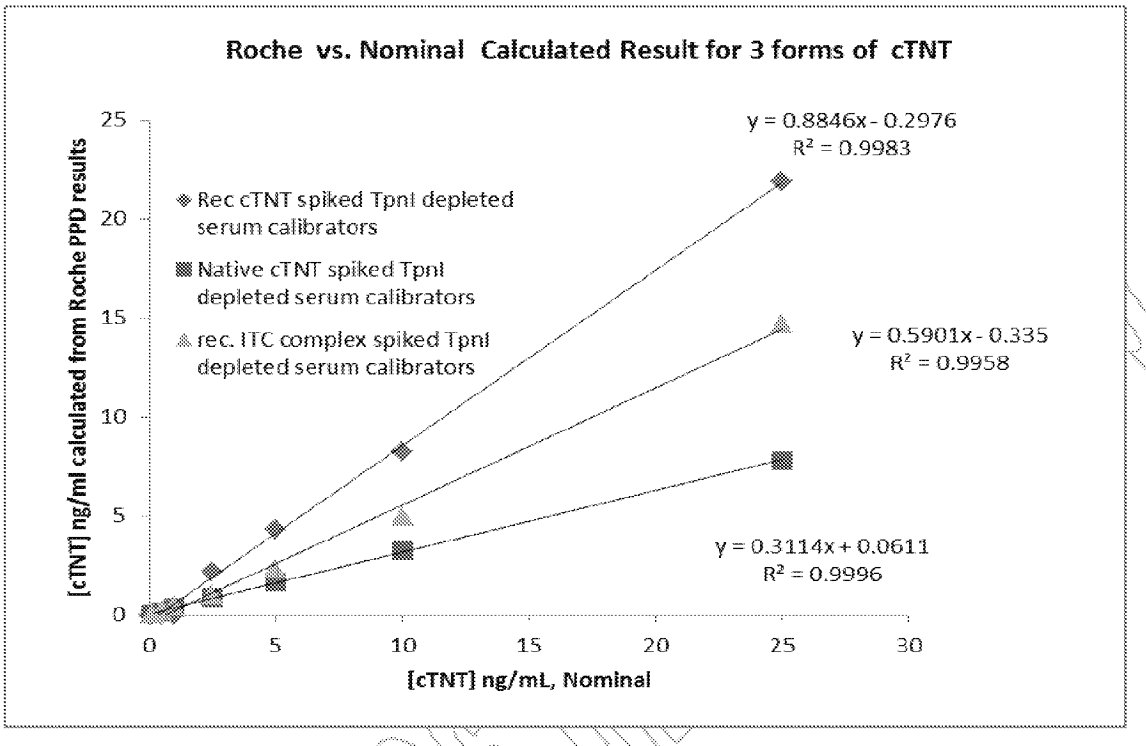
1.9 Calibrator comparison

Based on the conclusions from the above sections 25x stock solutions of the three forms of cTNT were formulated in citrate buffer and then spiked into troponin I depleted serum. These were tested on the Roche assay at an external site. The results are summarized in Table 14 and Figure 12. The recombinant cTNT gave the best correlation on the Roche assay. The native and ternary complex troponin T forms correlated poorly. The citrate formulation helps greatly to improve the correlation of the recombinant cTNT formulated in house to the Roche assay compared with data shown in Figure 1.

Table [SEQ Table * ARABIC]: Performance of Theranos cTNT calibrators on the Roche cTNT assay- Part 2

	Result from Roche assay		
Nominal [cTNT] ng/mL	ITC complex ng/mL	Native cTNT ng/mL	Rec cTNT ng/mL
25	14.7	7.8	21.89
10	5.0	3.3	8.26
5	2.3	1.7	4.33
2.5	1.0	0.9	2.17
1	0.4	0.4	0.90
0.5	0.2	0.2	0.48
0.1	0.04	0.03	0.09
0.025	OORL	OORL	0.02
0	OORL	OORL	OORL

Figure [SEQ Figure * ARABIC]: Performance of Theranos cTNT calibrators on the Roche cTNT assay- Part 2



1.10 Training Set revisited

The clinical correlation data described in section 1.8 was analyzed again this time accounting for the assigned concentrations of the three different calibrators based on the data from the Roche assay. The results are summarized on Table 15. The same data using calibration curves based on nominal concentrations is shown on Table 16. Figures 13 -15 represent the clinical correlation and the final training set data. The clinical correlation ranges between R^2 values of 0.55-0.58 among all 6 scenarios. It was also seen that the slope approached a value of 1.0 only when the native CTNT was used as the calibrator and the calibration was based on nominal concentrations. It was decided to pursue the native cTNT calibrators as the final calibrator material and in the absence of any other source for validating the concentrations of the calibrators it was further decided to use the nominal concentrations throughout the remainder of the assay development.

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Table [SEQ Table * ARABIC]: Clinical sample correlation generated using reassigned calibrator concentration

CTNT reported ng/mL PPD/ProMedDx	CTNT results ng/mL Roche/PPD	Matrix	Inter-Cartridge Mean	Inter-Cartridge CV%	Back calculated Conc . (ng/mL) rec CTNT Mean	rec CTNT CV%	rec CTNT % Recovery	native CTNT Mean	native CTNT CV%	native CTNT % Recove	rec ITC complex Mean	rec ITC complex CV%	rec ITC complex % Recovery
10.8	10.11	Serum	41549	10	2.90	9	29	2.20	10	22	2.52	11	25
8.22	7.75	Serum	49242	10	3.44	10	44	2.58	10	33	3.03	11	39
7.47	6.83	Serum	86808	4	5.97	4	87	4.65	4	68	5.82	5	85
4.84	4.65	Serum	10058	1	0.63	26	13	0.57	0	12	0.57	1	12
3.79	3.56	Serum	5575	14	0.36	19	10	0.29	18	8	0.29	17	8
3.56	3.37	Serum	14800	12	1.16	13	34	0.85	12	25	0.87	13	26
3.43	3.63	Li-Heparin plasma	37742	20	2.65	18	73	2.01	18	55	2.28	21	63
3.23	3.21	Li-Heparin plasma	13342	9	1.05	12	33	0.77	9	24	0.78	10	24
2.8	2.68	Li-Heparin plasma	13841	11	1.04	12	39	0.80	11	30	0.81	12	30
2.61	2.71	Li-Heparin plasma	3719	1	0.20	19	7	0.17	2	6	0.17	2	6
2.05	2.01	Li-Heparin plasma	19499	15	1.45	14	72	1.10	13	55	1.15	15	57
1.8	1.73	Li-Heparin plasma	10239	19	0.75	23	43	0.59	20	34	0.58	21	34
1.64	1.94	Li-Heparin plasma	9899	5	0.73	5	38	0.57	5	29	0.56	5	29
1.4	1.43	Li-Heparin plasma	5675	6	0.37	9	26	0.30	8	21	0.29	8	20
1.02	1.02	Li-Heparin plasma	1788	17	0.04	81	4	0.05	29	5	0.05	27	5
0.76	0.76	K3EDTA Plasma	2025	14	0.06	22	8	0.06	27	8	0.06	24	8
0.27	0.27	K3EDTa plasma	3539	16	0.19	25	69	0.15	24	57	0.15	22	57
0.05	0.05	K3EDTA	1060	18	0.02	47	32	0.01	45	28	0.02	39	37
0.03	0.03	K3EDTa plasma	563	10	OORL			OORL			OORL		
0.01	0.01	K3EDTa plasma	488	13	OORL			OORL			OORL		
0	0	K3EDTa plasma	448	4	OORL			OORL			OORL		

Table [SEQ Table * ARABIC]: Clinical sample correlation generated using nominal calibrator concentrations

CTNT reported ng/mL PPD/ProMedDx	Matrix	Inter-Cartridge Mean	Inter-Cartridge CV%	Back calculated Conc . (ng/mL) rec CTNT Mean	rec CTNT CV%	rec CTNT % Recovery	native CTNT Mean	native CTNT CV%	native CTNT % Recovery	rec ITC complex Mean	rec ITC complex CV%	rec ITC complex % Recovery
10.8	Serum	41549	10	3.40	10	32	6.41	10	59	5.23	8	48
8.22	Serum	49242	10	4.08	10	50	7.58	11	92	6.04	9	73
7.47	Serum	86808	4	7.23	4	97	14.29	5	191	10.51	4	141
4.84	Serum	10058	1	0.67	26	14	1.71	0	35	1.57	1	32
3.79	Serum	5575	14	0.38	18	10	0.88	18	23	0.78	19	21
3.56	Serum	14800	12	1.27	14	36	2.50	11	70	2.27	11	64
3.43	Li-Heparin plasma	37742	20	3.10	19	90	5.85	19	171	4.83	16	141
3.23	Li-Heparin plasma	13342	9	1.14	13	35	2.27	9	70	2.07	8	64
2.8	Li-Heparin plasma	13841	11	1.13	12	40	2.35	11	84	2.14	10	76
2.61	Li-Heparin plasma	3719	1	0.21	18	8	0.50	2	19	0.42	2	16
2.05	Li-Heparin plasma	19499	15	1.61	15	79	3.21	13	157	2.86	12	140
1.8	Li-Heparin plasma	10239	19	0.80	24	44	1.75	19	97	1.60	19	89
1.64	Li-Heparin plasma	9899	5	0.78	6	48	1.69	5	103	1.56	5	95
1.4	Li-Heparin plasma	5675	6	0.39	9	28	0.90	8	64	0.80	9	57
1.02	Li-Heparin plasma	1788	17	0.04	75	4	0.14	30	13	0.10	34	9
0.76	K3EDTA Plasma	2025	14	0.07	20	9	0.17	28	22	0.12	32	16
0.27	K3EDTa plasma	3539	16	0.20	24	73	0.46	24	171	0.38	27	142
0.05	K3EDTA	1060	18	0.02	43	42	0.04	49	74	0.02	57	40
0.03	K3EDTa plasma	563	10	OORL			OORL			OORL		
0.01	K3EDTa plasma	488	13	OORL			OORL			OORL		
0	K3EDTa plasma	448	4	OORL			OORL			OORL		

Figure [SEQ Figure * ARABIC]: Clinical correlation generated with Troponin ternary complex as calibrator material.

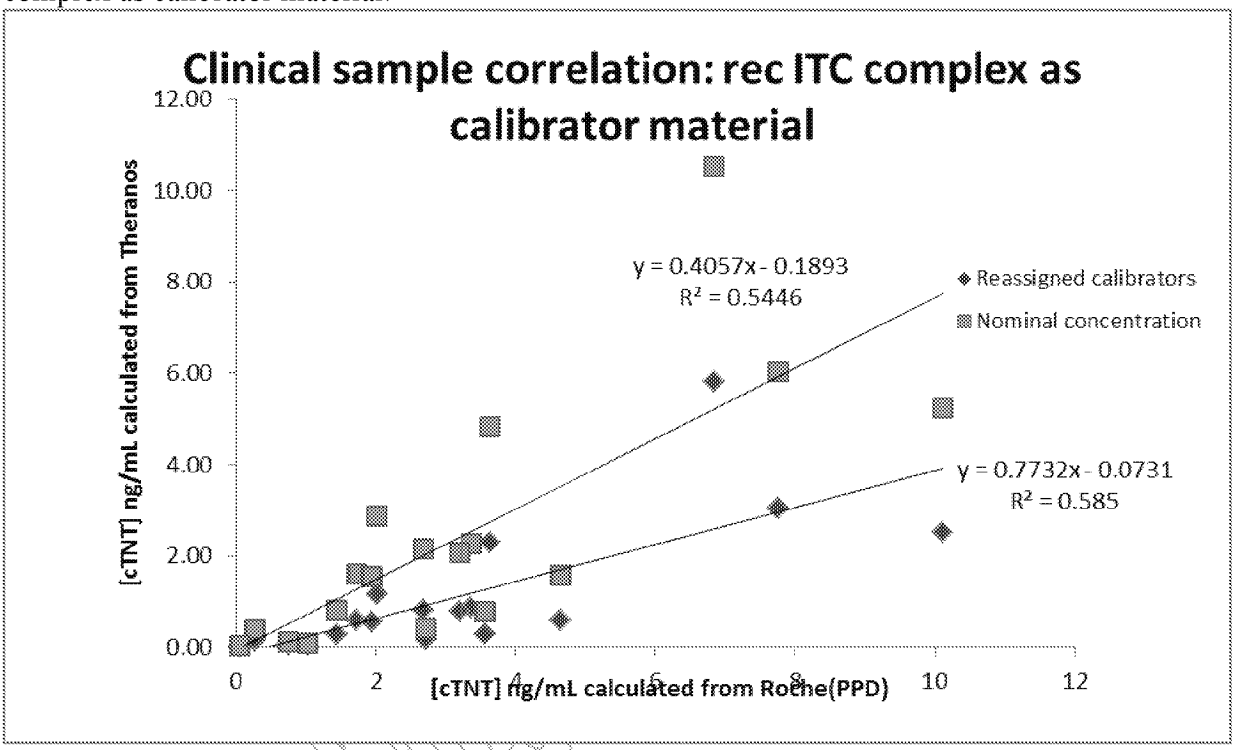


Figure [SEQ Figure * ARABIC]: Clinical correlation generated with native cTNT as calibrator material.

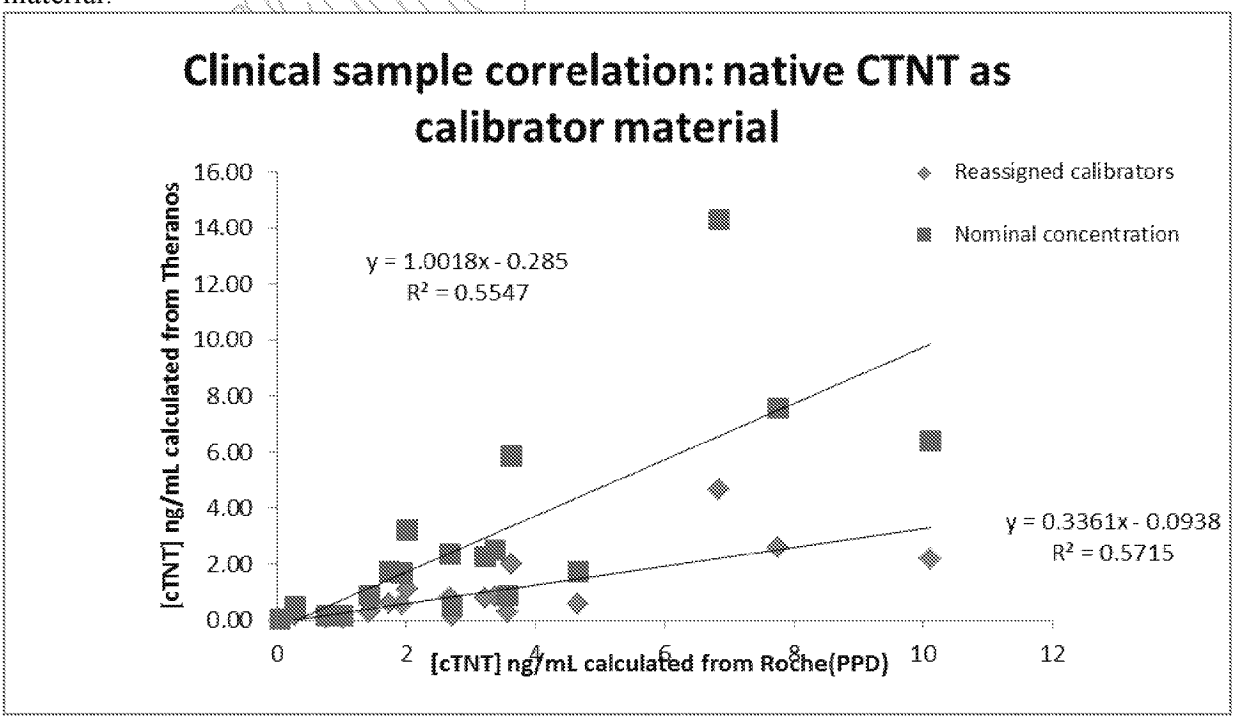
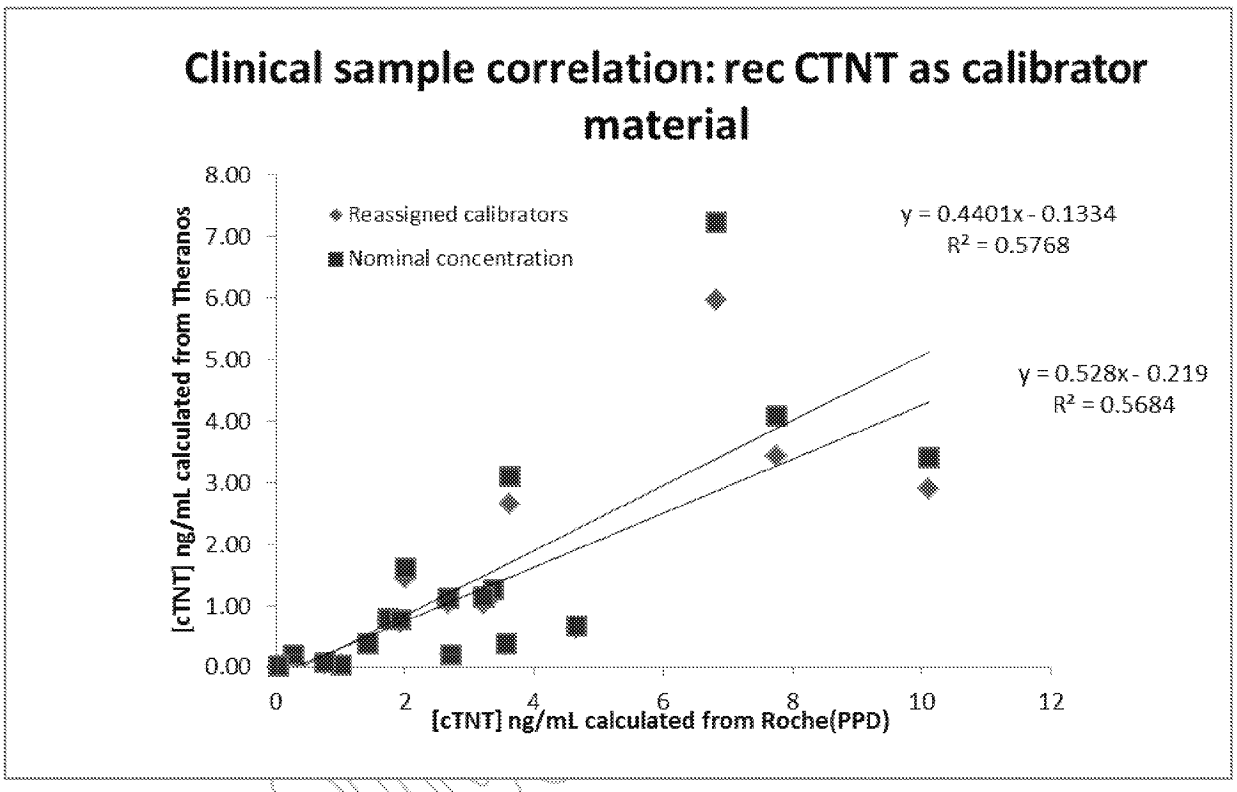


Figure [SEQ Figure * ARABIC]: Clinical correlation generated with rec cTNT as calibrator material.



1.11 Normal Serum and Plasma Screen

In the case of the third generation Roche cTNT assay, heparin plasma could not be recommended for cTNT determination owing to a direct interference with the immunoassay by heparin leading to systematic lower test results compared to serum results. In order to find out if such a matrix effect existed in the Theranos cTNT assay N=10 matched normal donor serum and Li-heparin plasma samples were tested. Table 17 shows the standard curve used and Table 18 summarizes the back calculated concentrations. As shown in figure 16 there is an excellent correlation between serum and Li-heparin samples (R62 = 0.87 and slope = 0.98). At this time it was concluded that the current Ab pair does not have any adverse matrix effects and Li-heparin samples could be used as test samples.

Table [SEQ Table * ARABIC]: Standard Curve native cTNT

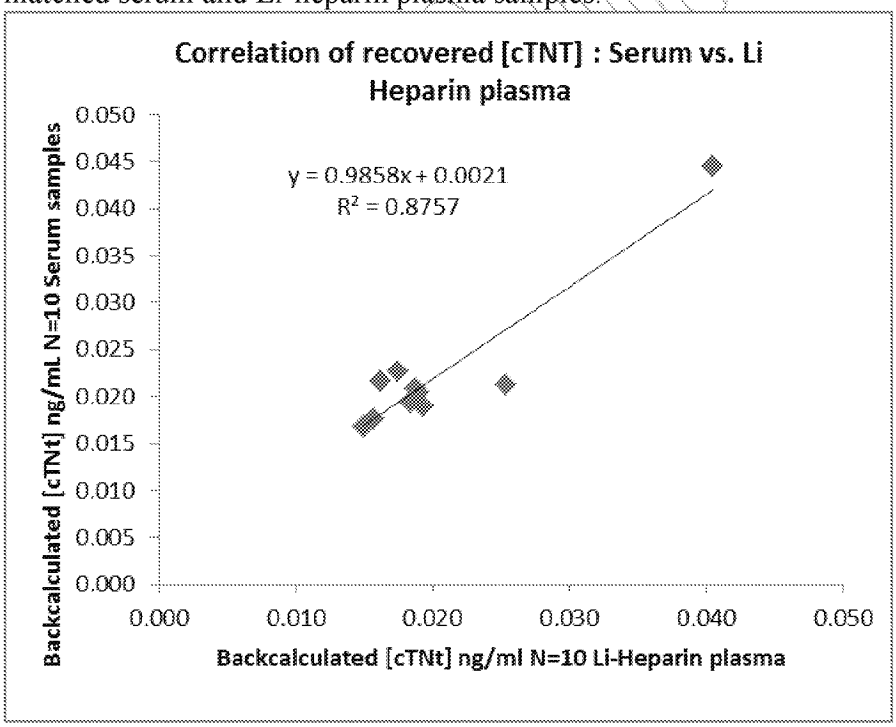
Nominal ng/mL	Inter-Cartridge Mean	CV%	S/B	Inter-Cartridge Mean	CV%	% Recovery
25	106499	15	380	22.57	12	90
10	39072	14	140	9.76	13	98
5	22886	6	82	5.86	6	117
2.5	11715	15	42	2.90	17	116
1	4798	17	17	1.02	21	102
0.5	2238	10	8	0.38	13	76
0.1	818	42	2.9	0.09	63	93
0.025	403	13	1.4	0.03	15	119
0	280	8	1.0	0.01	7	

Calibration curve: $y = -0.1777x^2 + 2.5463x - 6.9552$

Table [SEQ Table * ARABIC]: Back calculated cTNT concentrations of N=10 matched serum and Li-heparin plasma samples

Li-Hep Plasma	Inter-Cartridge RLU		Inter-Cartridge Conc (ng/mL)		Serum	Inter-Cartridge RLU		Inter-Cartridge Conc. (ng/mL)	
Sample ID	Mean	CV%	Mean	CV%	sample ID	Mean	CV%	Mean	CV%
P1	291	7	0.017	11	S1	341	9	0.023	14
P2	300	13	0.018	22	S2	309	18	0.019	28
P3	360	24	0.026	24	S3	331	11	0.022	18
P4	290	16	0.018	13	S4	293	12	0.018	20
P5	304	16	0.019	28	S5	320	5	0.020	8
P6	310	7	0.019	12	S6	289	17	0.017	11
P7	265	14	0.015	24	S7	284	10	0.017	16
P8	487	14	0.040	23	S8	517	12	0.044	14
P9	365	15	0.025	24	S9	327	14	0.021	22
P10	303	9	0.019	15	S10	307	16	0.019	10

Figure [SEQ Figure * ARABIC]: Correlation of back calculated cTNT concentrations: N=10 matched serum and Li-heparin plasma samples.



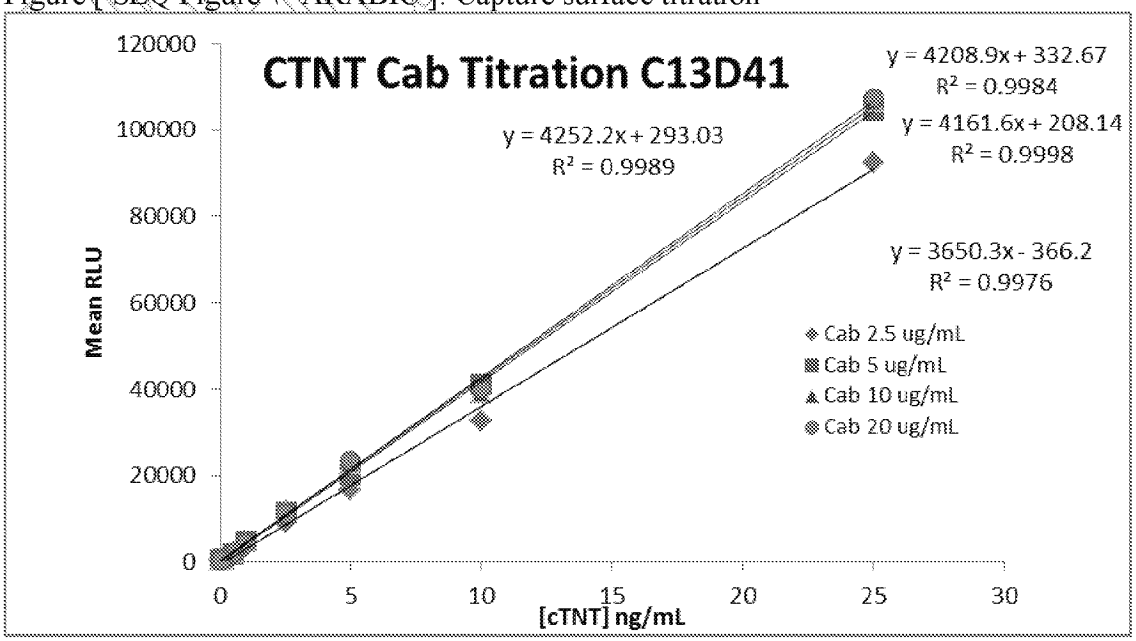
1.12 Capture Titration

The biotin conjugated anti-cTNT antibody was titrated at 4 levels to determine the ideal concentration to be used as capture coating surface. The calibrators used were native cTNT spiked into Troponin I depleted serum. The alkaline phosphatase labeled anti-cTNT antibody was mixed with the diluted sample at a final concentration of 5 ng/mL in Biostab in a homogeneous format. The final effective sample dilution was 25 fold. Table 19 summarizes the capture Ab titration results and they are depicted as a plot in Figure 17. The results showed that both 10 and 20 ug/mL concentrations of the capture antibody afforded good signal modulation and similar sensitivity at the low end. 10 ug/ml was finalized as the capture concentration for the cTNT assay.

Table [SEQ Table * ARABIC]: cTNT assay capture titration

	Cab 2.5 ug/mL		Cab 5 ug/mL		Cab 10 ug/mL		Cab 20 ug/mL	
Nominal	Inter-Cartridge		Inter-Cartridge		Inter-Cartridge		Inter-Cartridge	
ng/mL	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
25	92450	5	104578	8	106499	15	107215	8
10	32708	6	41157	7	39072	14	40303	21
5	16638	21	20242	20	22886	6	23455	14
2.5	9203	7	11509	9	11715	15	11031	12
1	3459	8	4643	9	4798	17	4548	18
0.5	1923	14	1822	27	2238	10	2228	8
0.1	638	9	723	17	968	27	817	37
0.025	411	10	476	19	403	13	401	13
0	346	9	353	7	280	8	278	5
S/B	267		297		380		386	
Low end modulation	1.19		1.35		1.4		1.4	

Figure [SEQ Figure * ARABIC]: Capture surface titration



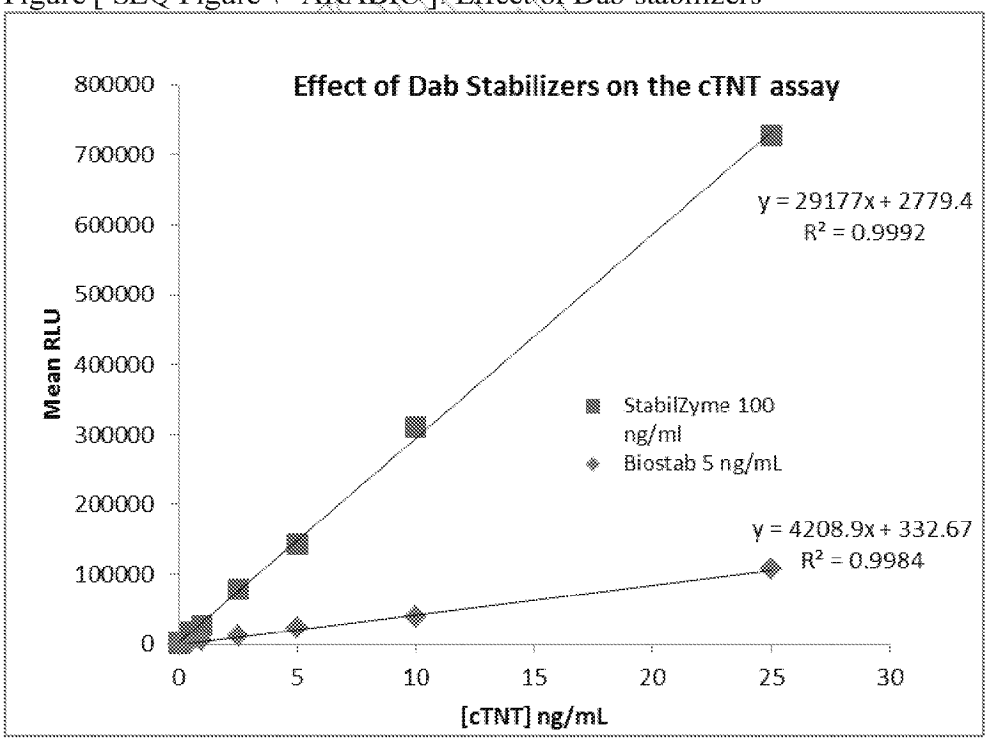
1.13 Effect of Detection Ab Stabilizers

Table 20 summarizes the result of this experiment. StabilZyme provided the best signal to background and improved sensitivity compared to Biostab and was chosen as the Dab stabilizer for the cTNT assay. Figure 18 shows the comparison of the dose response using these two commercially available alkaline phosphatase conjugate stabilizers.

Table [SEQ Table * ARABIC]: Effect of Dab stabilizers on the cTNT assay

	BIOSTAB			StabilZyme		
Nominal [cTNT] ng/mL	Inter-Cartridge Mean	CV%	S/B	Inter-Cartridge Mean	CV%	S/B
25	106499	15	380	726701	0	451
10	39072	14	140	311105	6	193
5	22886	6	82	142993	14	89
2.5	11715	15	42	77910	7	48
1	4798	17	17	26956	19	17
0.5	2238	10	8	17447	11	11
0.025	403	13	1.4	3197	13	2.0
0	280	8	1.0	1610	5	1

Figure [SEQ Figure * ARABIC]: Effect of Dab stabilizers



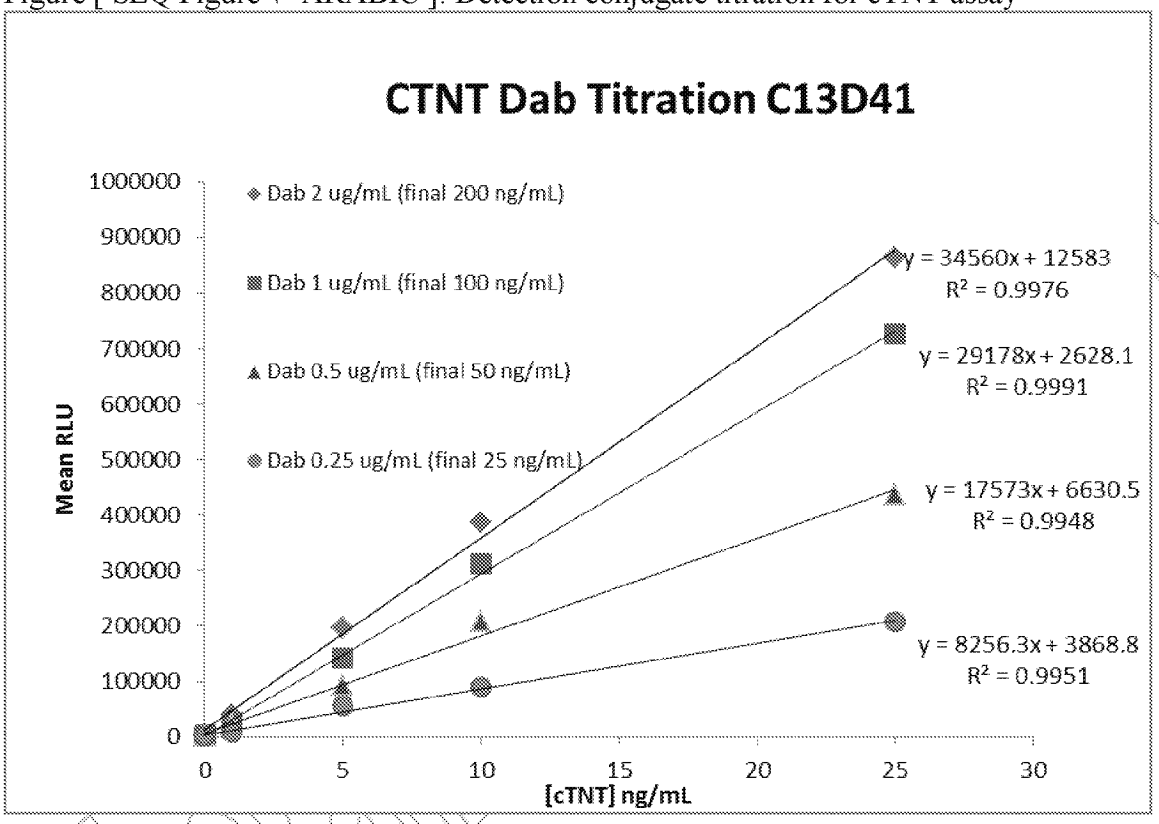
1.14 Detection Ab Titration

The alkaline phosphatase labeled anti-cTNT antibody was titrated at four levels in StabilZyme. Since a homogeneous assay format was used the loading concentration of the Dab was varied from 2 µg/ml to 0.25 µg/ml. The final concentrations of the Dab after a 10 fold dilution on board ranged from 200 -25 ng/mL. Based on the signal to background and sensitivity (Table 21, Figure 19) the Dab concentration was finalized at 1 µg/mL.

Table [SEQ Table * ARABIC]: Dab titration

Loading [Dab]	**2 ug/ml**			**1 ug/ml**		
Final [Dab] ng/mL	**200 ng/ml**			**100 ng/ml**		
Nominal [cTNT] ng/mL	**Inter-Cartridge Mean RLU**	**CV%**	**S/B**	**Inter-Cartridge Mean RLU**	**CV%**	**S/B**
25	863348	14	335	726701	0	393
10	386391	5	150	311105	6	168
5	196686	7	76	142993	14	77
1	39831	16	15	26956	19	15
0.025	4496	11	1.7	3197	13	2
0	2581	1	1	1847	9	1
Loading [Dab]	**0.5 ug/ml**			**0.25 ug/ml**		
Final [Dab] ng/mL	**50 ng/ml**			**25 ng/ml**		
Nominal [cTNT] ng/mL	**Inter-Cartridge Mean RLU**	**CV%**	**S/B**	**Inter-Cartridge Mean RLU**	**CV%**	**S/B**
25	436526	14	388	207153	5	362
10	206975	3	184	89527	3	156
5	93006	12	83	55301	2	97
1	21612	6	19	8567	2	15
0.025	1484	15	1.3	806	35	1.4
0	1124	9	1	573	6	1

Figure [SEQ Figure * ARABIC]: Detection conjugate titration for cTNT assay



1.15 Effect of Coating Buffer

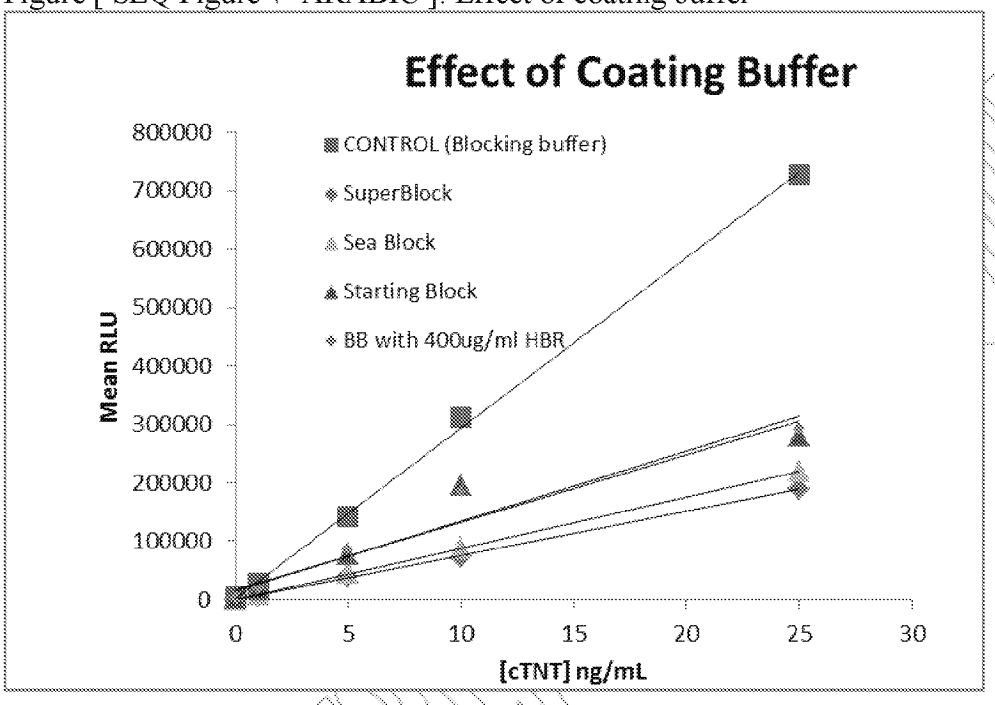
The effect of changing the coating surface buffer was tested. The control coating buffer used was 3% BSA/0.05% sodium azide in TBS. Three commercially available blockers: Super Block, Sea Block and Starting Block were tested against the control. A fourth blocker included 400 µg/ml of HBR (Scantibodies, Inc) in blocking buffer. All blockers resulted in a diminished dose response compared to control. Although the HBR reagent afforded a lower background it also lowered the signal at the top end. It was decided to proceed with blocking buffer as the final coating surface buffer.

Table [SEQ Table * ARABIC]: Effect of coating buffer

	Control (Blocking buffer)			SuperBlock			Sea Block		
Nominal	Inter-Cartridge RLU		S/B	Inter-Cartridge RLU		S/B	Inter-Cartridge RLU		S/B
ng/mL	Mean	CV%		Mean	CV%		Mean	CV%	
25	726701	0	393	188844	10	354	220221	6	300
10	311105	6	168	74003	7	139	87056	11	119
5	142993	14	77	38149	7	72	45926	10	63
1	26956	19	15	7332	7	14	9225	4	13
0.025	3197	13	1.7	674	38	1.3	842	9	1.1
0	1847	9	1	533	8	1	734	4	1

	Starting block			BB+400 ug/ml HBR		
Nominal	Inter-Cartridge RLU		S/B	Inter-Cartridge RLU		S/B
ng/mL	Mean	CV%		Mean	CV%	
25	279208	55	152	293316	54	214
10	195809	10	107	184622	13	135
5	77452	7	42	85044	8	62
1	17658	21	10	18419	19	13
0.025	3922	10	2.1	2348	6	1.7
0	1834	30	1	1372	12	1

Figure [SEQ Figure * ARABIC]: Effect of coating buffer



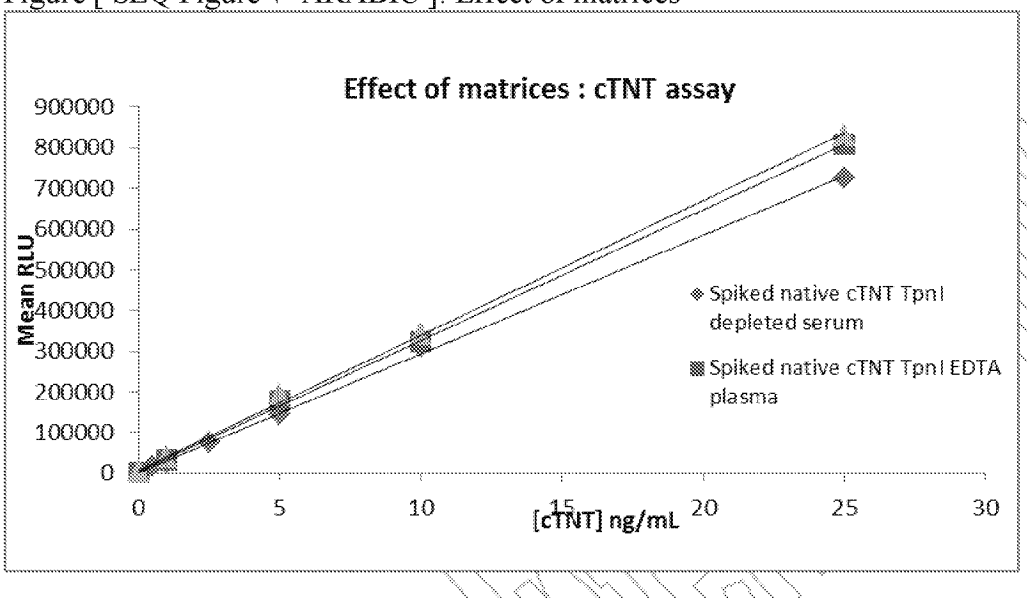
1.16 Effect of Matrices and Spike Recovery

The effect of different matrices (depleted serum, normal EDTA plasma and Li-heparin plasma) on the cTNT assay was investigated by spiking native cTNT into the above. In all cases the spiked recovery was within 20% of nominal. It was concluded that there is no undesirable matrix effect seen for this assay. Table 23 summarizes the matrix data and Figure 21 depicts the dose response in each of these matrices.

Table [SEQ Table * ARABIC]: Effect of matrices

Matrix	Nominal ng/mL	Signal (RLU) Mean	CV%	Conc (ng/mL) Mean	CV%	% Recovery
Depleted serum	25	726701	0	21.54	0	86
	10	311105	6	11.41	5	114
	5	142993	14	5.60	14	112
	2.5	77910	7	2.95	7	118
	1	26956	19	0.80	25	80
	0.5	17447	11	0.44	15	88
	0.025	3197	13	0.03	13	117
	0.0125	1984	9	0.01	35	108
	0	1610	5	0.01	2	
pooled EDTA plasma	25	808909	5	23.08	3	92
	10	324576	14	11.80	12	118
	5	144547	3	5.66	3	113
	1	32745	11	1.04	14	104
	0.025	3084	3	0.03	15	109
	0	2917	6	0.02	12	
pooled Li-Heparin plasma	25	829973	10	23.44	6	94
	10	343734	36	11.98	22	120
	5	149868	4	5.87	4	117
	1	29769	6	0.91	8	91
	0.025	3108	6	0.03	12	110
	0	3174	9	0.03	15	

Figure [SEQ Figure * ARABIC]: Effect of matrices



1.17 Effect of Anticoagulants

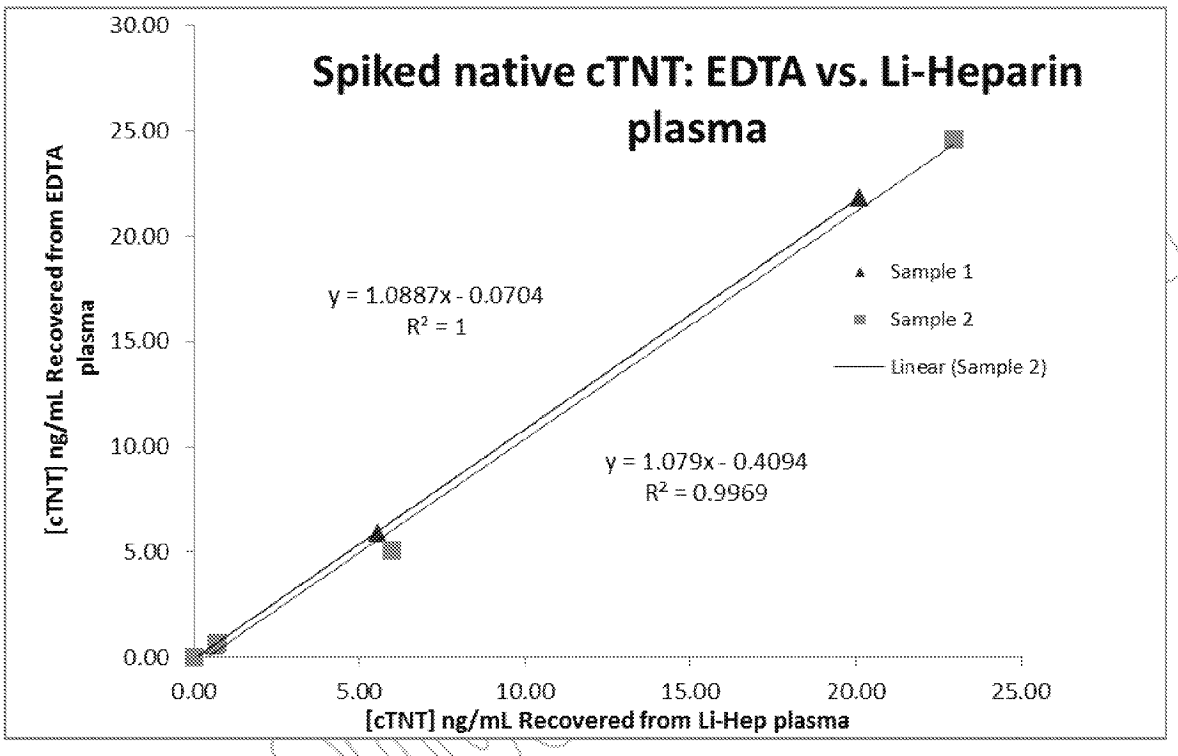
Whole blood sampels from 2 donors each collected in EDTA as well as heparin tubes was obtained from the Stanford donor bank. Each sample was spun down and the respectiove plasma was obtained. The endogenous cTNt levels were found to be 0.01 and 0.02 ng/mL respectively. cTNT was spiked into each of these plasma at 3 additional levels above endogenous. The back calculated concentrations and recoveries are tabulated on Table 24. The heparin and EDTA spiked cTNT data from each patient was correlated and the R^2 values were found to be close to 1.0. This indicated that there was no effect of either anti-coagulant on the assay. Figure 22 shows the correlation.

Table [SEQ Table * ARABIC]: Effect of anticoagulants

		Li- Heparin plasma					EDTA plasma			
Whole Blood Sample ID	**Nominal ng/mL**	Signal (RLU) **Mean**	**CV%**	**Conc. ng/mL Mean**	**CV%**	**% Recovery**	**Signal (RLU) Mean**	**CV%**	**Conc. ng/mL Mean**	**CV%**
1	25	656347	5	20.11	4	80	742266	4	21.84	3
	5	141534	3	5.55	3	111	151025	12	5.91	12
	0.5	22782	8	0.64	11	125	21637	21	0.60	29
	0	1509	8	0.01	16		1527	2	0.01	3
2	25	802048	8	22.95	5	92	894130	2	24.60	1
	5	152744	1	5.98	1	119	129689	1	5.08	1
	0.5	23993	37	0.69	50	133	23044	7	0.65	9
	0	2469	2	0.02	4		2827	12	0.02	22

7

Figure [SEQ Figure * ARABIC]: Effect of anticoagulants



1.18 Effect of Interfering Matrices

Hemolyzed, icteric and lipemic serum samples were obtained from a commercial source. The recovery of native cTNT spiked into these potentially interfering matrices was evaluated on the Theranos system. The spiked depleted serum calibration was used. Spike recoveries obtained for the hemolyzed and icteric samples were within 20% of nominal. The lipemic sample evaluated had a high triglyceride level of 270 mg/dL. Recovery of cTNT from this sample was poor. It was concluded that grossly lipemic samples would interfere with the cTNT assay. A similar observation was also made for the Roche assay.

Table [SEQ Table * ARABIC]: Effect of interfering matrices

Interfering Matrix (Serum)	Nominal [cTNT] ng/mL	RLU		Conc. ng/mL		% Recovery
		Mean	CV%	Mean	CV%	
Hemolyzed	25	941529	12	25.34	8	101
	10	307745	0	11.32	0	111
	5	149258	22	5.83	21	112
	1	33668	3	1.07	4	89
	0.1	13315	13	0.30	19	99
	0	10387	2	0.20	3	
Icteric	25	908916	6	24.83	3	99
	10	300722	3	11.10	3	110
	5	168803	3	6.59	3	129
	1	37177	21	1.22	26	109
	0.1	12879	8	0.28	12	128
	0	7583	7	0.12	11	
Lipemic	25	576639	7	18.38	5	73
	10	246606	11	9.34	10	93
	5	126990	1	4.98	1	99
	1	18868	29	0.50	39	49
	0.1	3944	18	0.04	31	38
	0	1821	10	0.01	19	

1.19 Rf Positive and HAMA Positive Sample Screen

6 HAMA positive and 6 Rf positive serum samples were screened for levels of cTNT. The purpose of the screen was to determine if these samples interfered and gave a false positive result for the assay. The results are summarized in Table 26. All samples tested showed OORL (out of range low) result on the assay.

Table [SEQ Table * ARABIC]: Rf and HAMA positive sample screen

Interfering Matrix (Serum)	Sample ID	Result ng/mL	RLU Mean	RLU CV%	Conc. Mean ng/mL
HAMA positive	2	48.0	1304	7	OORL
	3	68.1	1111	4	OORL
	4	54.9	1363	4	OORL
	6	45.4	2480	0	OORL
	8	40.0	1511	10	OORL
	9	41.2	1500	9	OORL
Rf positive	304	168	2598	8	OORL
	305	153	1423	4	OORL
	663	190	1376	9	OORL
	321	162	927	3	OORL
	676	142	1268	7	OORL
	658	186	1902	16	OORL

1.20 Effect of assay diluent

Further attempts were made to improve accuracy and precision at the bottom end of the assay. The target LLOQ, 0.025 ng/mL, could only be claimed if the recovery at that concentration was within 20%. Data using the standard in house diluent : 3% BSA /0.05% sodium azide tended to increase background depending on the lot of BSA used. It was decided to switch diluent to a commercial blocker, Starting Block and evaluate the sensitivity and accuracy at the lower end of the calibration curve. An anchor point at the low end of the curve at 0.0125 ng/mL was introduced and the in house calibration software was applied to compute the LLOQ and ULOQ for the assay along with accuracy and precision at these levels. Table 27 compares the responses between blocking buffer and Starting Block as assay diluents. Clearly Starting Block as the assay diluent decreased background and improved accuracy the low end of the curve. It was decide to switch out the assay diluent to Starting Block at this point of the assay validation

Table [SEQ Table * ARABIC]: Effect of assay diluent

Assay Diluent	Blocking buffer			Starting Block		
Nominal ng/mL	RLU Mean	CV%	S/B	RLU Mean	CV%	S/B
25	717735	7	383	290956	17	345
10	291437	2	155	120986	10	144
5	154893	5	83	62924	8	75
2.5	74326	3	40	33265	10	39
1	21880	19	12	13266	8	16
0.5	16523	2	9	8092	16	10
0.1	5508	12	3	2261	5	3
0.025	3012	5	1.6	1330	9	1.6
0.0125	1956	13	1.0	1087	7	1.3
0	1876	9	1	843	7	1

1.21 Final calibration

The following assay conditions were finalized for the cTNT assay: Capture antibody concentration of 10 µg/mL in blocking buffer, Starting Block as assay diluent, detection conjugate concentration of 1 ug/mL (loading) in StabilZyme and 100 ng/mL after dilution. The effective sample dilution was set at 25 fold. The assay format was a 5x5 homogeneous format where the sample was diluted and mixed with the detection conjugate and incubated on the capture surface for 5 minutes followed by wash and incubation of the chemiluminescent substrate for additional 5 minutes, followed by a read of the RLU. Native cTNT spiked into troponin I depleted serum was used as the calibrator material. A single lot of reagents were prepared and the final calibration curve was run using a 10 point calibrator curve. The results are summarized in Table 28.

Table [SEQ Table * ARABIC]: . cTNT calibration curve

[cTNT] ng/ml	Signal (RLU) Mean	CV%	S/B	Conc. ng/mL Mean	CV%	% Recovery
25	290956	17	332	24.0	19	96
10	120986	10	138	9.4	10	94
5	62924	8	72	4.9	8	98
2.5	33265	10	38	2.6	10	104
1	13266	8	15	1.0	9	99
0.5	8092	16	9	0.6	19	113
0.1	2261	5	3	0.1	9	89
0.025	1307	16	1.5	0.021	19	83
0.0125	1117	5	1.3	OORL		
0	876	13	1	OORL		

LLOQ	0.025 ng/mL
ULOQ	25 ng/mL
Curve fit	Log Lin 4PL
Accuracy at LLOQ	83%
Precision at LLOQ	19%
Accuracy at ULOQ	106%
Precision at ULOQ	3%

Conc = 4.444 * (((6.810-2.704) / (log10 (RLU) - 2.704)) - 1) ^ (1 / (-0.427))

1.22 Normal Sample (N=50) Screen and Matched Pair Analyses

The recommended biochemical marker for the diagnosis and risk assessment of patients with myocardial infarction by the European Society of Cardiology and the American College of Cardiology is a rise and fall of cardiac troponin (cTnT) with at least one value above the 99th percentile of a reference population the upper reference limit (URL), and the assays used should have an imprecision, coefficient of variation (CV) of 10% at this cut-off point. In order to ascertain the 99th percentile cutoff a reference population of normal cardi healthy donors is desired. This was accomplished by testing N=50 normal donor samples from the Stanford blood bank, comprising of N=25 males and N-=25 females. Each donor sample was collected in serum separation, EDTA and heparin vacutainers and the corresponding serum/plasma was obtained. All samples were run on the Theranos cTNT assay. Due to the difficulty in obtaining matched samples across the range of the assay a matched pair analysis was conducted with this sample set. The results are summarized in Table 29 and Table 30.

Table [SEQ Table * ARABIC]: Results from N=25 male normal donor screen

	Serum				EDTA plasma				% Diff					% Diff
Sample	RLU		Conc. ng/ml		RLU		Conc. ng/ml		from	RLU		Conc. ng/ml		from
ID	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%	serum	Mean	CV%	Mean	CV%	serum
M1	1162	1	OORL	-	1052	4.1	OORL	-		1128	1	OORL	-	
M2	1132	6	OORL	-	1092	1.6	OORL	-		1173	5	OORL	-	
M3	1098	5	OORL	-	1213	7.4	OORL	-		1138	4	OORL	-	
M4	1381	0	0.03	1	1387	1.0	0.030	-	-1	1562	3	0.026	7	-15
M5	1023	1	OORL	-	1195	3.0	OORL	-		1127	7	OORL	-	
M6	1098	1	OORL	-	1119	0.7	OORL	-		1055	8	OORL	-	
M7	1133	3	OORL	-	1306	0.0	OORL	-		1352	2	OORL	-	
M8	1375	0	0.030	0	1410	1.2	0.032	3	7	1435	0	OORL	-	
M9	1375	4	0.030	-	1288	2.5	0.025	7	-17	1467	1	OORL	-	
M10	1501	4	0.037	9	1278	3.7	OORL	-		1679	3	0.031	9	-17
M11	1015	5	OORL	-	928	2.4	OORL	-		1143	1	OORL	-	
M12	1044	3	OORL	-	1027	1.1	OORL	-		1160	10	OORL	-	
M13	1148	6	OORL	-	1301	0.2	OORL	-		1351	0	0.026	1	
M14	1243	2	OORL	-	1271	6.2	OORL	-		1318	8	OORL	-	
M15	1158	7	OORL	-	1299	0.8	OORL	-		1287	3	OORL	-	
M16	1217	3	0.021	8	1308	0.4	0.026	1	9	1357	4	OORL	-	
M17	1107	3	OORL	-	1287	0.6	0.025	2		1519	8	OORL	-	
M18	1006	4	OORL	-	1058	8.3	OORL	-		1264	4	OORL	-	
M19	2021	6	0.071	11	2006	0.6	0.070	1	-1	2282	2	0.065	4	-8
M20	1142	3	OORL	-	1039	1.0	OORL	-		1063	12	OORL	-	
M21	969	5	OORL	-	1067	3.0	OORL	-		1180	1	OORL	-	
M22	1072	1	OORL	-	1101	5.3	OORL	-		1120	6	OORL	-	
M23	1102	5	OORL	-	1091	7.5	OORL	-		1194	3	OORL	-	
M24	1018	5	OORL	-	1029	6.8	OORL	-		1336	0	OORL	-	
M25	1319	1	0.027	2	1437	2.5	0.033	6	24	1504	7	0.033	6	25

Table [SEQ Table * ARABIC]: Results from N=25 female normal donor screen

	Serum				EDTA plasma				% Diff					% Diff
Sample	RLU		Conc. ng/ml		RLU		Conc. ng/ml		from	RLU		Conc. ng/ml		from
ID	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%	serum	Mean	CV%	Mean	CV%	serum
F1	1098	3	OORL	-	1036	1	OORL	-		1095	12	OORL	-	
F2	1076	4	OORL	-	1020	1	OORL	-		986	1	OORL	-	
F3	1221	0	OORL	-	1218	5	OORL	-		1262	3	OORL	-	
F4	1179	1	OORL	-	1044	6	OORL	-		1258	3	OORL	-	
F5	1448	7	0.034	18	1392	4	0.031	10	-10	1658	7	0.030	19	-12
F6	1392	8	0.031	19	1304	4	0.026	10	-16	1509	9	OORL	-	
F7	1459	8	0.031	13	1322	5	0.027	13	-14	1694	2	0.032	6	1
F8	1343	3	0.030	4	1275	3	0.024	7	-19	1688	1	0.031	2	5
F9	1077	3	OORL	-	1245	4	OORL	-		1213	1	OORL	-	
F10	1101	0	OORL	-	1211	7	OORL	-		1214	2	OORL	-	
F11	1133	19	OORL	-	1187	5	OORL	-		1213	7	OORL	-	
F12	1106	28	0.026	6	1181	7	OORL	-		1353	0	OORL	-	
F13	1056	15	OORL	-	982	2	OORL	-		910	4	OORL	-	
F14	989	4	OORL	-	973	1	OORL	-		1096	6	OORL	-	
F15	1243	5	0.026	7	1260	8	OORL	-		1546	2	0.025	6	-4
F16	1075	24	OORL	-	1348	5	OORL	-		1466	10	OORL	-	
F17	1069	21	OORL	-	1036	4	OORL	-		1162	7	OORL	-	
F18	1310	3	0.026	9	1363	3	0.029	8	11	1685	1	0.031	2	19
F19	1309	11	OORL	-	1232	2	OORL	-		1248	0	OORL	-	
F20	1396	6	0.033	6	1381	1	0.030	3	-9	1597	4	0.027	11	-17
F21	1370	4	0.030	10	1297	6	0.025	16	-14	1741	2	0.034	5	14
F22	1371	5	0.027	2	1323	6	0.027	17	0	1467	2	OORL	-	
F23	1450	8	0.031	8	1408	6	0.032	15	3	1595	2	0.027	5	-12
F24	1313	2	0.026	7	1363	4	0.029	10	11	1594	2	0.027	5	2
F25	1396	3	0.029	9	1285	2	0.025	5	-15	1611	3	0.028	7	-5

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1.23 Cross reactivity

Cross reactivity of the CTNT assay towards skeletal cTNT, Troponin C, Troponin I and Troponin I-C binary complex was evaluated by spiking two clinical samples (with known levels of cTNT) with 50 ng/mL of these analytes. The back calculated concentrations were calculated and the percentage of cross reactivity was computed using the formula: (([cTNT spiked]-[cTNT unspiked]) /50 ng/ml)*100. All of the above analytes had no significant cross reactivity with the Ab pair chosen for the cTNT assay. Table 31 summarizes the cross reactivity data.

Table [SEQ Table * ARABIC]: Cross reactivity

	Serum sample FS21					Serum sample MS19				
Cross reactive analyte	**RLU**		**Conc.(ng/mL)**		**% Cross Reactivity**	**RLU**		**Conc.(ng/mL)**		**% Cross Reactivity**
	Mean	**CV%**	**Mean**	**CV%**		**Mean**	**CV%**	**Mean**	**CV%**	
cTNC spiked @ 50 ng/ml	1121	7	0.02	5	0.00	1985	14	0.08	28	0.00
Unspiked	1091	14	0.02	17		2136	15	0.08	29	
Sk. cTNT spiked @ 50 ng/ml	1094	2	0.02	7	0	2787	16	0.13	27	0.12
Unspiked	763	2	OORL			1966	6	0.07	13	
cTNI spiked @ 50 ng/ml	934	6	OORL		0.00	2000	14	0.07	27	0.04
Unspiked	999	9	OORL			1665	3	0.05	7	
I-C complex spiked @ 50 ng/ml	1176	3	OORL		0.00	1636	9	0.05	20	0.00
Unspiked	1120	14	OORL			2181	13	0.08	25	

1.24 Clinical sample validation

35 samples were tested as the final set of clinical samples for validating the cTNt assay. These sampels were tested on the Roche cTNt assay at an external facility and the results were compared with the Theranos assay results. The clinical correlation was improved compared to the training set data (R^2= 0.74 and slope = 0.95). This might indicate that sample heterogeneity might play a role in this assay. Table 32 summarizes the standard curve and Table 33 summarizes the clinical sample data. Figure 22 depicts the clinical correlation.

Table [SEQ Table * ARABIC]: Standard curve for cTNT assay

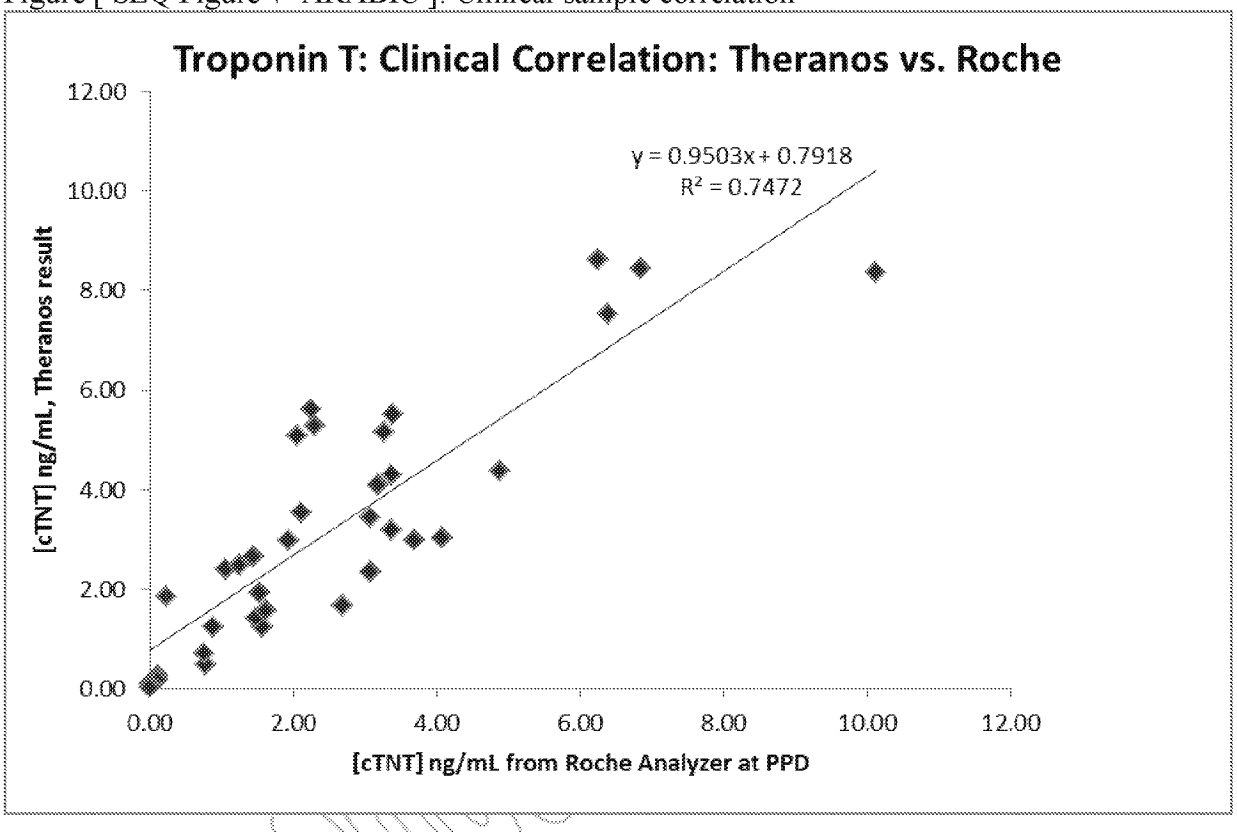
	Signal (RLU)			Conc. ng/mL		% Recovery
[cTNT] ng/ml	**Mean**	**CV%**	**S/B**	**Mean**	**CV%**	
25	290956	17	332	24.0	19	96
10	120986	10	138	9.4	10	94
5	62924	8	72	4.9	8	98
2.5	33265	10	38	2.6	10	104
1	13266	8	15	1.0	9	99
0.5	8092	16	9	0.6	19	113
0.1	2261	5	3	0.1	9	89
0.025	1307	16	1.5	0.021	19	83
0.0125	1117	5	1.3	OORL		
0	876	13	1	OORL		

LLOQ	0.025 ng/mL
ULOQ	25 ng/mL
Curve fit	Log Lin 4PL
Accuracy at LLOQ	83%
Precision at LLOQ	19%
Accuracy at ULOQ	106%
Precision at ULOQ	3%

Table [SEQ Table * ARABIC]: Clinical Sample correlation: Theranos vs. Roche cTNT assays

	Sample ID	Roche analyzer (ng/mL)	Theranos CTNT assay result
1	11691824	0.78	0.49
2	11691829	1.56	1.26
3	11692389	0.76	0.72
4	11692394	1.47	1.43
5	11692400	1.61	1.56
6	11692401	4.88	4.37
7	11692414	3.38	5.51
8	11692419	3.06	3.44
9	11692424	3.27	5.15
10	11692435	1.43	2.66
11	11692440	3.18	4.10
12	11692443	3.07	2.35
13	BRH515315	6.38	7.53
14	BRH515316	3.37	3.20
15	BRH515317	2.68	1.66
16	BRH515318	3.69	3.00
17	BRH515319	4.07	3.03
18	11101173	0.10	0.26
19	11265401	0.10	0.19
20	11306164	0.00	0.10
21	11308033	0.23	1.84
22	11522317	0.00	0.03
23	11523423	6.25	8.61
24	11661558	2.10	3.55
25	11640719	2.05	5.07
26	11482617	2.30	5.28
27	11461454	2.24	5.62
28	11398310	1.53	1.92
29	11372860	1.05	2.42
30	11445037	0.88	1.25
31	11663212	10.11	8.36
32	11661416	6.83	8.44
33	11662944	3.37	4.30
34	11461431	1.93	2.99
35	11397876	1.25	2.47

Figure [SEQ Figure * ARABIC]: Clinical sample correlation



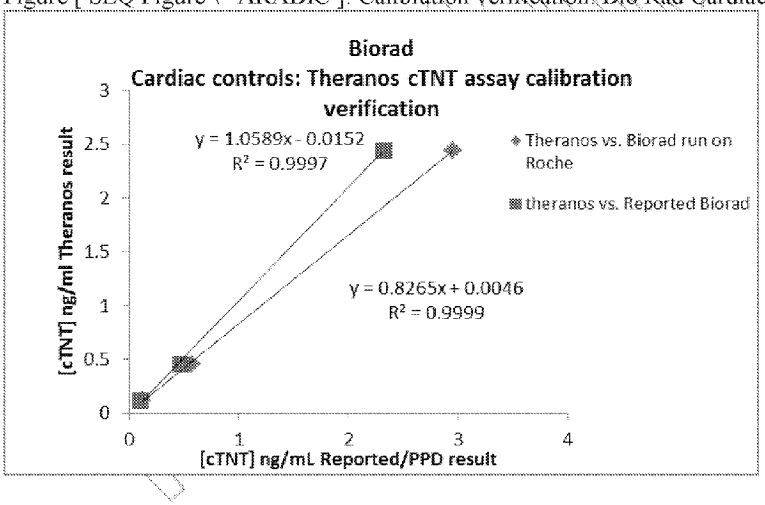
1.25 Calibration verfication: Biorad cardiac Controls

The assay calibration was verified by testing commercially available cardiac control samples at three levels. The results showed good correlation with reported data for these controls. Data are summarized in Table 34 and Figure 24.

Table [SEQ Table * ARABIC]: Calibration verification: BioRad Cardiac controls

	[cTNT] in Bio Rad Cardiac Controls, ng/ml		RLU		Conc		% Recovery
Sample ID	**PPD/Roche analyzer results**	**Reported conc on datasheet**	**Mean**	**CV%**	**Mean**	**CV%**	
					ng/ml		
Level 1	0.12	0.107	2645	14	0.12	24	98
Level 2	0.57	0.47	6831	6	0.46	7	81
Level 3	2.95	2.32	31429	19	2.45	20	83

Figure [SEQ Figure * ARABIC]: Calibration verification: Bio Rad Cardiac controls



1.26 Effect of lower sample dilution

In an effort to see if the LLOQ can be lowered to < 0.025 ng/mL assay conditions that afforded lower sample dilution and higher reagent incubation time were tested. Decreasing the sample dilution 5 fold from 25x to 5x and increasing the reagent incubation time in the homogeneous format from 5_5 to 10_10 helped to decrease the LLOQ from 25 to 12.5 pg/mL (0.0125 ng/mL). The results are summarized in Table 35. The 5x sample dilution at 10_10 reagent incubation was chosen as the final condition for the cTNT assay and the calibration exercise was performed (see Table as the The clinical sample validation was repeated at these new assay conditions and the results tracked with the previous validation data (shown in Table 38).

Table [SEQ Table * ARABIC]: Effect of lower sample dilution

	5x_coincubation_5_5			5x_coincubation_10_10			10x_coincubation_5_5		
[cTNT] ng/ml	Inter-Cartridge Mean	CV%	S/B	Inter-Cartridge Mean	CV%	S/B	Inter-Cartridge Mean	CV%	S/B
25	551908	13	688	1042280	8	777	286617	25	304
15	349550	13	436				160428	7	170
10	206181	6	257	446292	0	333	113793	21	121
5	113202	7	141				52865	19	56
2.5	53209	20	66	125947	1	94	33286	15	35
1	21314	16	27				8558	83	9
0.5	10275	12	13	25101	4	19	6070	2	6
0.25	6646	4	8				3774	10	4
0.1	3465	4	4.3	5197	6	4	2436	23	2.6
0.05	1703	14	2.1	3664	1	2.7	1347	18	1.4
0.025	1420	6	1.8	2948	7	2.2	1132	10	1.2
0.0125	1225	2	1.5	2308	2	1.7	942	13	1.0
0.00625	915	17	1.1	1822	12	1.4	1021	28	1.1
0	802	14	1.0	1342	1	1	980	25	1

Table [SEQ Table * ARABIC]: Calibration with revised assay conditions

	Inter-Cartridge RLU			Inter-Cartridge Conc.		% Recovery
[cTNT]	Mean	CV%	S/B	Mean	CV%	
ng/ml				ng/ml		
25	1042280	8	777	24.7	10	99
10	446292	0	333	9.4	0	94
2.5	125947	1	94	2.6	1	105
0.5	25101	4	19	0.5	4	107
0.1	5197	6	4	0.081	13	81
0.05	3664	1	2.7	0.048	2	96
0.025	2948	7	2.2	0.030	18	120
0.0125	2308	2	1.7	0.017	9	137
0.00625	1822	12	1.4	OORL		
0	1342	1	1	OORL		
LLOQ	0125 ng/mL					
ULOQ	25 ng/mL					
Curve fit	4PL					
Accuracy at LLOQ	102%					
Precision at LLOQ	12.5%					
Accuracy at ULOQ	102%					
Precision at ULOQ	5.5%					

Table [SEQ Table * ARABIC]: Clinical sample validation under revised assay conditions

Sr. No.	Sample ID	Reported	Inter-Cartridge RLU		Inter-Cartridge Conc.	
		(ng/mL)	Mean	CV%	Mean	CV%
					ng/ml	
2	11691829	1.56	190549	16	3.9	16
3	11692389	0.76	104645	7	2.2	6
4	11692394	1.47	168871	26	3.5	25
5	11692400	1.61	231983	14	4.8	14
6	11692401	4.88	286218	12	5.9	12
14	BRH515316	3.37	107524	7	2.2	7
15	BRH515317	2.68	213302	25	4.4	25
16	BRH515318	3.69	457681	4	9.6	5
19	11265401	0.01	2821	11	0.027	30
22	11522317	0	3069	3	0.033	6
23	11523423	6.25	526543	10	11.2	12
31	11663212	10.8	711085	6	15.6	7

Figure [SEQ Figure * ARABIC]: Clinical Sample validation