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B-type Natriuretic Peptide (BNP) 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"]

B-type Natriuretic Peptide (BNP), also known as brain natriuretic peptide, comprises a 32 amino acid peptide with a 17 amino acid ring closed by a disulfide bond between two cysteine residues. Its amino acid sequence is highly conserved across species. BNP is synthesized and secreted from the ventricle of the heart into the circulation in response to ventricular stretching and volume load. Increased BNP concentrations are associated with heart failure [1].

This assay is designed to quantitatively determine the concentration of BNP in EDTA anticoagulated plasma.

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

Alere Biosite Triage BNP test provided by CLIA Lab has been used as a predicate method.

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

Biotinylated Anti-BNP Fab 106.3 from Binder Group serves as the capture surface. The sample is diluted and mixed with mouse anti-human BNP detection antibody conjugated to alkaline phosphatase, and the reaction mixture is incubated on the capture surface. The surface is then washed. The alkaline phosphatase substrate is incubated on the surface, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials and Reagents

Name	Supplier	Catalog #
Biotinylated Anti-BNP Fab 106.3	In-house Binder Group	Batch #2
Mouse Anti-Human BNP Antibody	LifeSpan BioSciences	LS-B7942
Alkaline Phosphatase Labeling Kit (SH)	Dojindo	LK13
Alkaline Phosphatase Substrate	In-house	Lot#19032013GT-ALP-A
Starting Block Buffer	Thermo Scientific	37542
Theranos Small Molecule AP Stabilizer	In-house	

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

2.1 Capture and Detection Antibody Pairs Screen on MTP

In-house and commercial biotinylated antibodies (Table 2) were coated on 384-well plates (20ug/mL UA precoated) at 10ug/mL in 3% BSA blocking buffer and screened with BNP Controls level 1-3 from Thermo Scientific and detection antibodies conjugated with alkaline phosphatase. Some promising pairs from MTP screening were shown in Table 3

Table [SEQ Table * ARABIC]: Anti-BNP Antibody Information

Dab#	Description	Vendor	Cat #
B1	Rabbit anti-human Brain Natriuretic Peptide (aa 1-10) antibody	Immundiagnostik	AE1034.2
B4	Mouse anti-human Brain Natriuretic Peptide (aa 1-32) antibody (Clone #: 21-46)	Immundiagnostik	A 1042.2
B5	Mouse anti-human Brain Natriuretic Peptide (aa 1-32) antibody(Clone #: 17-16)	Immundiagnostik	A 1041.2
B6	Mouse anti-human Brain Natriuretic Peptide (aa 1-32) antibody (Clone #: 3-21)	Immundiagnostik	A 1044.2
B7	BNP antibody (5-E2)	Novus	NB100-62133
B8	NPPB Mouse anti-Human Monoclonal (50E1) Antibody	LifeSpan BioSciences	LS-C51845
B9	NPPB Mouse anti-Human Monoclonal (24C5) Antibody	LifeSpan BioSciences	LS-C51846
B10	NPPB Mouse anti-Human Monoclonal (26E2) Antibody	LifeSpan BioSciences	LS-C51849
B11	NPPB Mouse anti-Human Monoclonal (50B7) Antibody	LifeSpan BioSciences	LS-C51843-200
B12	NPPB Mouse anti-Human Monoclonal (57H3) Antibody	LifeSpan BioSciences	LS-B7942
B13	Rabbit anti-Brain Natriuretic Peptide (BNP)	US Biological	N0520-20
B14	Mouse anti-Brain Natriuretic Peptide (BNP) (5E509)	US Biological	B2702-29B
B15	Mouse anti-Brain Natriuretic Peptide (BNP) (5E513)	US Biological	B2702-29F
B16	Mouse anti-Brain Natriuretic Peptide (BNP) (5E515)	US Biological	B2702-29H
B17	Mouse anti-Brain Natriuretic Peptide (BNP) (10B316)	US Biological	B2702-29T
B18	Mouse anti-Brain Natriuretic Peptide (BNP) (10B317)	US Biological	B2702-29U
Binder	Biotinylated Anti-BNP Fab 106.3	In-house Binder	

Table [SEQ Table * ARABIC]: Promising Pairs from MTP Screening

DAB	CAB	[BNP] pg/mL	Mean	CV %	Modulation
B4	B10	Level 3	13106	7	8
		Level 2	15874	33	10
		Level1	6377	138	4
		0	1631		
B7	B9	Level 3	11836	31	14
		Level 2	2848	2	3
		Level1	1071	31	1
		0	874		
B7	B10	Level 3	2098	9	70
		Level 2	1253	1	42
		Level1	948	8	32
		0	30		
B7	B15	Level 3	14633	1	16
		Level 2	3107	4	3
		Level1	1121	2	1
		0	934		
B7	B16	Level 3	17054	7	18
		Level 2	3801	14	4
		Level1	629	49	1
		0	946		
B7	B18	Level 3	17826	1	15
		Level 2	3119	12	3
		Level1	1349	31	1
		0	1208		
B7	Binder Ab	Level 3	18447	3	16
		Level 2	4285	27	4
		Level1	1390	40	1
		0	1188		
B4	B10	Level 3	13106	7	8
		Level 2	15874	33	10
		Level1	6377	138	4
		0	1631		
B8	B16	Level 3	3871	6	5
		Level 2	1080	4	1
		Level1	713	14	1
		0	771		

DAB	CAB	[BNP] pg/mL	Mean	CV %	Modulation
B8	Binder Ab	Level 3	7646	59	11
		Level 2	1117	7	2
		Level1	834	44	1
		0	702		
B9	B12	Level 3	7053	10	16
		Level 2	2741	11	6
		Level1	887	11	2
		0	432		
B11	B10	Level 3	60582	13	17
		Level 2	3377	2	1
		Level1	4409	2	1
		0	3477		
B12	CAB B15	Level 3	63940	0	23
		Level 2	18589	14	7
		Level1	6421	5	2
		0	2737		
B12	CAB B18	Level 3	66279	6	17
		Level 2	22027	12	6
		Level1	7622	11	2
		0	4000		
B12	Binder	Level 3	75379	3	14
		Level 2	21594	2	4
		Level1	7557	19	1
		0	5317		
B15	CAB B11	Level 3	20640	2	12
		Level 2	6484	3	4
		Level1	2483	7	1
		0	1670		
B15	CAB B14	Level 3	13201	2	6
		Level 2	7181	15	3
		Level1	2469	15	1
		0	2230		

2.2 Capture and Detection Antibody Pairs Screen on Theranos

Ten antibody pairs from MTP screening were tested on Theranos Analyzer using Thermo Scientific BNP Controls. Four antibody pairs were excluded from further development due to low modulation. The remaining six antibody pairs were continued to test on Theranos Analyzer using BNP calibrators prepared in 3% BSA and BNP & NT-pro BNP depleted Plasma. Finally, five clinical samples were tested with these antibody pairs. The results showed that the modulation was very low for all six antibody pair.

Table [SEQ Table * ARABIC]: Antibody Pairs Tested on Theranos Analyzer Using BNP Controls (Thermo Scientific)

DAB	CAB	[BNP] pg/mL	Mean	CV %	Modulation
B4	B10	Level 3	42183	19	1
		Level 2	64303	12	1
		Level1	38307	21	1
		0	50157	11	
B7	B10	Level 3	2473	15	2
		Level 2	1489	37	1
		Level1	1372	23	1
		0	1304	22	
B7	Binder	Level 3	4327	38	5
		Level 2	1553	33	2
		Level1	614	18	1
		0	835	15	
B7	B15	Level 3	7820	33	12
		Level 2	2159	10	3
		Level1	590	7	1
		0	655	13	
B7	B18	Level 3	8550	30	14
		Level 2	1334	28	2
		Level1	609	38	1
		0	631	16	
B8	Binder	Level 3	544	7	1
		Level 2	653	12	1
		Level1	1129	12	1
		0	775	16	
B11	B10	Level 3	26155	86	12
		Level 2	5850	35	3
		Level1	2760	82	1
		0	2184	26	
B12	Binder	Level 3	9567	26	7
		Level 2	2429	24	2
		Level1	796	13	1
		0	1397	26	
B12	B15	Level 3	7751	18	8
		Level 2	2038	33	2
		Level1	1233	17	1
		0	974	33	
B12	B18	Level 3	6818	31	6
		Level 2	1883	33	2
		Level1	1429	19	1

		0	1222	17	

Table [SEQ Table * ARABIC]: Antibody Pairs Tested on Theranos Analyzer Using Calibrators Prepared in 3% BSA

DAB	CAB	Nominal [BNP] pg/mL	Mean	CV %	Modulation
B7	B15	500	138584	27	252
		250	37038	25	67
		50	1254	23	2
		0	550	30	
B7	B18	500	153031	28	191
		250	24862	32	31
		50	1155	8	1
		0	800	11	
B11	B10	500	183541	44	87
		250	65138	46	31
		50	4005	27	2
		0	2108	37	
B12	Binder	500	58849	19	42
		250	17987	15	13
		50	1885	6	1
		0	1410	15	
B12	B15	500	75530	9	87
		250	19398	18	22
		50	748	8	1
		0	870	7	
B12	B18	500	103760	2	78
		250	16855	23	13
		50	1291	27	1
		0	1325	15	

Table [SEQ Table * ARABIC]: Antibody Pairs Tested on Theranos Analyzer Using Calibrators Prepared in BNP & NT-pro BNP Depleted Plasma

DAB	CAB	Nominal [BNP] pg/mL	Mean	CV %	Modulation
B7	B15	500	22326	6	15
		250	5625	3	4
		50	2524	8	2
		0	1523	17	
B7	B18	500	17630	9	9
		250	10073	19	5
		50	1846	18	1
		0	1993	11	
B11	B10	500	34982	27	21
		250	6170	26	4
		50	1237	15	1
		0	1702	6	
B12	Binder	500	13075	22	29
		250	6068	22	13
		50	1217	12	3
		0	452	12	
B12	B15	500	7957	15	13
		250	4300	27	7
		50	801	22	1
		0	597	21	
B12	B18	500	9991	24	15
		250	3503	4	5
		50	991	9	1
		0	666	2	

Table [SEQ Table * ARABIC]: Antibody Pairs Tested on Theranos Analyzer with Clinical Samples

DAB	CAB	Clinical Sample#	Biosite Triage (pg/mL)	Mean	CV %	Modulation
B7	B15	15	566	1398	12	3
		13	282	617	27	1
		12	118	628	10	1
		9	53.8	495	20	1
		N9	15.5	550	19	
B7	B18	15	566	1139	31	2
		13	282	650	21	1
		12	118	564	23	1
		9	53.8	584	4	1
		N9	15.5	534	11	
B11	B10	15	566	2281	45	3
		13	282	698	20	1
		12	118	800	18	1
		9	53.8	602	19	1
		N9	15.5	759	17	
B12	Binder	15	566	2507	2	4
		13	282	1342	18	2
		12	118	985	18	2
		9	53.8	797	26	1
		N9	15.5	565	16	
B12	B15	15	566	1540	19	3
		13	282	731	14	1
		12	118	596	18	1
		9	53.8	441	3	1
		N9	15.5	511	11	
B12	B18	15	566	1232	12	3
		13	282	703	19	2
		12	118	762	10	2
		9	53.8	799	14	2
		N9	15.5	449	18	

Since all the antibody pairs showed very low modulation with clinical samples, a coincubation protocol with sample dilution at 1:10 was tested using Thermo Scientific BNP Controls. The results showed dramatically improved modulation with all the antibody pairs. Then the coincubation protocol was tested with calibrators prepared in BNP & NT-pro BNP depleted Plasma.

Table [SEQ Table * ARABIC]: Antibody Pairs Tested with Coincubation Protocol Using Thermo Scientific BNP Controls

DAB	CAB	Thermo BNP Control	Mean	CV %	Modulation
B7	B15	Level 3 (335 pg/mL)	20503	31	51
		0	402	26	
B7	B18	Level 3 (335 pg/mL)	18123	11	41
		0	446	15	
B11	B10	Level 3 (335 pg/mL)	45813	48	81
		0	569	34	
B12	Binder	Level 3 (335 pg/mL)	7410	11	16
		0	477	3	
B12	B15	Level 3 (335 pg/mL)	8438	32	18
		0	463	29	
B12	B18	Level 3 (335 pg/mL)	11994	6	31
		0	384	15	

Table [SEQ Table * ARABIC]: Antibody Pairs Tested with Coincubation Protocol Using Calibrators Prepared in BNP & NT-pro BNP Depleted Plasma

DAB	CAB	Nominal [BNP] pg/mL	Assigned to CLIA (pg/mL)	Mean	CV %	Modulation
B11	Binder	5000	1460	24985	16	38
		500	235	2057	27	3
		100	52.3	1843	37	3
		50	27.8	661	23	1
		0	6	654	31	
B11	B10	5000	1460	13010	17	26
		500	932	845	28	2
		100	235	1251	33	3
		50	52.3	550	33	1
		0	6	496	33	
B12	Binder	5000	1460	17827	26	64
		2500	932	8367	55	30
		500	235	1907	48	7
		100	52.3	618	35	2
		50	27.8	513	26	2
		0	6	279	10	
B12	B15	5000	1460	16331	32	70
		2500	932	10539	27	45
		500	235	1312	54	6
		100	52.3	493	63	2
		50	27.8	466	39	2
		0	6	233	22	
B12	B18	5000	1460	23655	12	48
		100	52.3	496	36	1
		0	6	496	32	
B12	B10	5000	1460	2111	65	4
		100	52.3	401	66	1
		0	6	542	23	

Based on the results from coincubation protocol test with calibrators prepared in BNP & NT-pro BNP depleted plasma, two antibody pairs (binder capture & DAB B12, CAB B15 & DAB B12) were selected for further development based on the good modulation.

2.3 Diluent Test

3% BSA, Starting Block and Protein-Free Blocking Buffers were tested with Thermo Scientific Controls to see whether different diluent can improve the modulation. Starting Block gave the best modulation. Then Super Block and Starting Block were tested with clinical samples to confirm Starting Block is the best. Based on the results, Starting Block was selected as final diluent due to better signal modulation.

Table [SEQ Table * ARABIC]: Diluent Test using Thermo Scientific BNP Control

BNP, pg/mL	3% BSA Buffer			Starting Block			Protein-Free Blocking Buffer (Pierce)		
	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
335	7410	11	16	4239	6	33	3356	13	17
0	477	3		130	19		193	11	

Table [SEQ Table * ARABIC]: Diluent Test Using Clinical Samples

Binder CAB, DAB B12		Starting Block			Super Block		
Sample #	CLIA Lab(pg/mL)	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
11	708	4951	2	38	2951	28	7
20	135	953	14	7	817	24	2
7	66.1	632	4	5	593	16	2
17	<5.0	499	38	4	481	24	1
0 (BNP-depleted plasma)		130	19		395	23	

CAB B10, DAB B11		Starting Block			Super Block		
Sample #	CLIA Lab(pg/mL)	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
11	708	14946	36	51	33050	30	16
20	135	1333	21	5	2021	26	1
7	66.1	918	28	3	963	30	0
17	<5.0	649	37	2	562	26	0
0 (BNP-depleted plasma)		295	39		2044	14	

2.4 Cross Reactivity and Interference

Four cross reactants had been tested on Theranos Analyzer for cross reactivity and interference at four different levels with two candidate antibody pairs: binder capture antibody, DAB B12 and CAB B15, DAB B12. The results showed that all four cross reactants had no cross reactivity and interference with Theranos BNP Assay for both antibody pairs. Therefore, Binder capture and DAB B12 was selected as final antibody pair since in-house antibody has the preference for assay development if it is similar to commercial antibody.

Table [SEQ Table * ARABIC]: Cross Reactivity and Interference Test with Two Antibody Pairs (binder CAB, DAB B12; CAB B15, DAB B12)

A. Binder CAB, DAB B12

Test Substance	Nominal [BNP] pg/mL	[Test Substance] pg/mL	Mean RLU	CV%	Back-Calculated Conc, pg/mL		
					Mean Conc	CV%	%Recovery
NT-Pro BNP	5000	1000	14158	11	3601	8	72
	1000	1000	2780	11	698	14	70
	100	1000	648	17	81	29	81
	0	1000	416	51	41	82	
Pro-BNP	5000	1000	19592	14	4521	10	90
	1000	1000	2930	17	746	21	75
	100	1000	793	37	118	54	118
	0	1000	657	33	86	48	
VIP	5000	1000	16177	5	3980	4	80
	1000	1000	4105	23	1111	26	111
	100	1000	641	15	80	26	80
	0	1000	353	22	28	40	
ACTH	5000	1000	19147	32	4380	23	88
	1000	1000	3812	32	1018	37	102
	100	1000	794	24	115	38	115
	0	1000	279	56	20	98	

B. CAB B15, DAB B12

Test Substance	Nominal [BNP] pg/mL	[Test Substance] pg/mL	Mean RLU	CV%	Back-Calculated Conc, pg/mL		
					Mean Conc	CV%	%Recovery
NT-Pro BNP	5000	1000	19366	27	5173	22	103
	1000	1000	2693	31	828	34	83
	100	1000	614	21	113	35	113
	0	1000	392	32	54	67	
Pro-BNP	5000	1000	16153	21	4482	18	90
	1000	1000	2855	15	887	17	89
	100	1000	639	33	122	49	122
	0	1000	534	30	91	50	
VIP	5000	1000	17658	32	4798	26	96
	1000	1000	2765	25	854	28	85
	100	1000	680	21	133	31	133
	0	1000	453	21	68	38	
ACTH	5000	1000	17368	24	4747	20	95
	1000	1000	3226	33	1008	36	101
	100	1000	499	20	80	33	80
	0	1000	372	1	43	30	

2.5 Training Set

15 clinical plasma samples from Bioreclamation were tested on Theranos Analyzer using Binder capture and DAB12 antibody pair. These samples were also tested on Alere Biosite Triage provided by CLIA Lab to evaluate the clinical correalation. The results showed an excellent clinical correaltion between Theranos Analyzer and Alere Biosite Triage.

Table [SEQ Table * ARABIC]: Clinical Samples Tested on Theranos Analyzer (Training Set)

	Theranos Result, pg/mL			
Clinical Plasma Sample #	**Mean Conc**	**CV%**	**Biosite Triage, pg/mL**	**Reported (pg/mL)**
1	17	44	22.5	53
2	16	42	55.1	90
3	64	23	30.1	84
4	92	20	299	393
5	33	36	107	204
6	90	18	259	431
7	223	12	480	885
8	796	22	841	1932
9	203	24	306	735
S2	507	22	812	n/a
S3	546	20	693	n/a
S5	27	16	39.2	n/a
S6	61	42	33.6	n/a
S8	2192	5	2230	n/a
S10	57	19	133	n/a

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

[SHAPE * MERGEFORMAT]

2.6 Alkaline Phosphatase Stabilizers

Commercial and in-house alkaline phosphatase stabilizers were tested as detection antibody diluent. In comparison with all the other AP Stabilizers, Theranos Small Molecule AP Stabilizer consisting of 5 mM Mg^{2+}, 0.1 mM Zn^{2+}, and 0.03% BSA in TBS gave the best signal modulation.

Table [SEQ Table * ARABIC]: Alkaline Phosphatase Stabilizers

AP Stabilzer	[BNP] pg/mL	Mean RLU	CV%	Modulation
Starting Block	1460	27260	16	60
	932	9520	13	21
	108	1325	16	3
	0	453	45	
Biostab	1460	6181	24	16
	932	3322	14	9
	108	585	21	2
	0	381	27	
Starting Block spiked with Zn^{2+} & Mg^{2+}	1460	26048	20	45
	932	7814	15	13
	108	893	18	2
	0	583	32	
Theranos Small Molecule AP Stabilizer	1460	33485	25	71
	932	12106	26	26
	108	1420	15	3
	0	469	42	
Stabilzyme AP	1460	8771	24	19
	932	4996	20	11
	108	1249	22	3
	0	471	34	
Theranos AP Stabilizer	1460	14873	22	22
	932	8824	16	13
	108	1376	22	2
	0	679	46	

2.7 Capture Antibody Titration

The coating concentration of Binder Biotinylated BNP antibody Fab 106.3 was titrated at 2.5, 5, 10, 20 ug/mL in 3% BSA buffer. 10ug/mL was selected as capture antibody coating concentration based on the results.

Table [SEQ Table * ARABIC]: Capture Antibody Titration

[CAb] ug/mL	[BNP] pg/mL	Mean RLU	CV%	Modulation
20	1460	9980	11	37
	932	4647	9	17
	375	1126	15	4
	108	587	7	2
	52.3	413	15	2
	0	272	16	
10	1460	17686	6	42
	932	5991	18	14
	375	1424	16	3
	108	1057	15	3
	52.3	628	18	2
	0	417	13	
5	1460	13169	14	26
	932	5536	9	11
	375	1453	13	3
	108	750	17	1
	52.3	604	19	1
	0	502	14	
2.5	1460	8358	12	20
	932	4385	24	10
	375	1156	19	3
	108	834	27	2
	52.3	584	19	1
	0	425	14	

2.8 Detection Antibody Titration

The DAb B12 was titrated in Theranos Small Molecule AP Stabilizer (5mM Mg^{2+}, 0.1mM Zn^{2+}, and 0.03% BSA in TBS) at 1000, 500, 100 ng/mL loading concentration (final concentration is 100, 50, 10 ng/mL respectively due to 1:10 dilution of the antibody in the protocol). The modulation was the best with the DAb concentration at 1000 ng/mL loading. Therefore, 1000 ng/mL was selected as the final loading DAb concentration.

Table [SEQ Table * ARABIC]: Detection Antibody Titration

Loading [DAb] ng/mL	Final [DAb] in Sample Mixture, ng/mL	[BNP] pg/mL	Mean RLU	CV%	Modulation
1000	100	1460	33485	25	71
		932	12106	26	26
		108	1420	15	3
		0	469	42	
500	50	1460	12692	27	35
		932	6596	22	18
		108	979	22	3
		0	360	19	
100	10	1460	5748	24	22
		932	2541	20	10
		108	578	33	2
		0	263	5	

2.9 Reagent Incubation Time

The effect of shorter reagent incubation time was tested with sample reaction mixture and substrate incubation time respectively at 10, 10; 5, 5 and 2, 1 minutes. With shorter incubation time, the modulation was reduced. Therefore, the 10,10 minutes incubation was selected as the final condition.

Table [SEQ Table * ARABIC]: Reagent Incubation Time

Incubation Time, Min	[BNP] pg/mL	Mean RLU	CV%	Modulation
10, 10	1460	17686	6	42
	932	5991	18	14
	375	1424	16	3
	108	1057	15	3
	52.3	628	18	2
	0	417	13	
5, 5	1460	6853	12	31
	932	2379	10	11
	375	1126	21	5
	108	383	27	2
	52.3	190	18	1
	0	222	18	
2, 1	1460	798	18	7
	932	416	13	4
	375	258	9	2
	108	167	7	1
	52.3	147	27	1
	0	117	20	

2.10 Sample Dilution

The effect of sample dilution was tested with final sample dilution factors of 1:25, 1:10 and 1:5 into Starting Block Buffer. When the sample dilution was down to 1:5, the modulation was the best. Therefore sample dilution at 1:5 was selected as the final condition.

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

Sample Dilution	[BNP] pg/mL	Mean RLU	CV%	Modulation
1:25	1460	4252	32	10
	932	3267	11	8
	375	1462	25	4
	108	560	19	1
	52.3	539	27	1
	0	408	21	
1:10	1460	17686	6	42
	932	5991	18	14
	375	1424	16	3
	108	1057	15	3
	52.3	628	18	2
	0	417	13	
1:5	1460	29696	20	81
	932	12548	14	34
	375	4949	27	13
	108	1110	23	3
	52.3	644	12	2
	0	367	32	

2.11 Binder Capture Antibody Lot Comparison

Three lots of binder anti-BNP Fab 106.3 (Biotin form) antibodies were received from Binder group. The antibody concentration for lot #1, #2 and #3 is 1.4, 1.9 and 3.2 mg/mL respectively. All three lots of antibodies gave similar modulation. The lot 2 antibody was used for Tray QC and subsequent tests since lot #1 was used up by the time finishing the protocol optimization.

Table [SEQ Table * ARABIC]: Three lots of Binder Capture FAbs

Binder Lot #	[BNP] pg/mL	Mean RLU	CV%	Modulation
1	1460	11011	7	38
	932	3750	9	13
	375	1771	24	6
	108	702	28	2
	52.3	447	21	2
	0	290	48	
2	1460	11547	27	35
	932	4693	16	14
	375	1916	6	6
	108	677	18	2
	52.3	496	23	2
	0	327	21	
3	1460	8637	17	39
	932	3600	24	16
	375	1847	7	8
	108	700	19	3
	52.3	345	15	2
	0	222	33	

2.12 Cross Reactivity

To further confirm that the final condition with the final antibody pair (binder capture and DAB B12) has no cross reactivity with more cross reactants, total 9 cross reactants were tested on Theranos Analyzer. The results showed that there is no cross reactivity with all the tested cross reactants for Theranos BNP assay.

Table [SEQ Table * ARABIC]: Cross Reactivity

[BNP] pg/mL	Cross Reactants	Mean RLU	CV%	Mean Conc
0	ACTH (1000 pg/mL)	481	36	OORL
0	VIP (1000 pg/mL)	438	56	OORL
0	NT-Pro BNP (1000 pg/mL)	304	35	OORL
0	Pro BNP (1000 pg/mL)	549	33	OORL
0	Renin (50 ng/mL)	489	44	OORL
0	Angiotensin I (600 pg/mL)	487	26	OORL
0	ANF (1000pg/mL)	464	48	OORL
0	Endothelin I (20 pg/mL)	630	25	OORL
0	CNP-53 (1000 pg/mL)	287	48	OORL

2.13 Determination of LLOQ and ULOQ

A lot of reagents were produced and a calibration was performed using the final assay conditions of 1000 ng/mL (final 100 ng/mL) DAB B12 in Theranos Small Molecule AP Stabilizer, 10ug/mL Binder capture antibody with UA coat, and a 1:5 sample dilution with 3 cartridges per point. The protocol for Theranos 3.0 System is Generic2_5x_Coincubation (Incubation time is 10,10 minutes in this protocol).

A set of calibrators were run on the Theranos System 3.0 and proprietary calibration software was used to determine the LLOQ and ULOQ using FDA guideline for ELISA assay calibration. The LLOQ was 27.8 pg/mL, and the ULOQ was 5300 pg/mL. The back-calculation formula was applied for all the following tests.

Table [SEQ Table * ARABIC]: Standard Curve

Calibrator	Signal, RLU		Back-Calculated Conc, pg/mL		
[BNP] (pg/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
5300	333169	16	5414	14	102
2460	116285	17	2482	11	101
1460	57494	38	1587	22	109
932	20954	25	875	15	94
375	5563	21	373	15	99
108	1610	12	120	16	111
52.3	1190	22	66	27	126
27.8	721	32	OORL		
12.9	423	9	OORL		
0	485	18	OORL		

Table [SEQ Table * ARABIC]: Dexter Results for LLOQ and ULOQ

Measurement	Value	Units
LLOQ	27.80	pg/mL
ULOQ	5300.00	pg/mL
LLOQ accuracy	111	%
LLOQ precision	7.1	%
Average Residuals	19	%
Error in prediction: Best case	13	%
Error in prediction: Expected	17	%

2.14 Clinical Samples

42 clinical plasma samples obtained from Bioreclamation were tested on Theranos System 3.0, and calculated with the calibration mentioned above. These samples were also tested on Alere Biosite Triage provided by CLIA Lab to evaluate the clinical correalation. The good clinical correlation was shown in Figure 2.

Table [SEQ Table * ARABIC]: Clinical Samples

Clinical Plasma Sample #	Theranos	Biosite Triage (pg/mL)
1	OORL	22.5
2	128	55.1
3	OORL	30.1
4	90	299
5	OORL	107
6	93	259
7	226	480
8	796	841
9	197	306
10	OORL	< 5.0
11	OORL	< 5.0
12	OORL	< 5.0
13	OORL	< 5.0
14	OORL	49.3
15	OORL	179
16	OORL	70.8
17	OORL	63
18	OORL	27.5
19	28	51
20	369	617
21	29	145
22	40	108
23	OORL	49.2
24	OORL	101
25	26	267
26	137	333
27	OORL	11
28	OORL	83
29	OORL	20
S2	530	812
S3	671	693
S5	OORL	39.2

Clinical Plasma Sample #	Theranos	Biosite Triage (pg/mL)
S6	48	33.6
S8	2987	2810
S10	56	133
S11	530	1120
S12	OORL	163
S13	2886	3410
S14	885	769
S15	2288	>5000
S16	398	959
S17	578	996

Figure [SEQ Figure * ARABIC]: Clinical Correlation
[SHAPE * MERGEFORMAT]

2.15 Whole Blood, Plasma and Serum Screen

Ten whole blood samples (samples F1-F5 from female, samples M1-M5 from male) were screened on Theranos Analyzer. EDTA plasma samples, Lithium-Heparin plasma samples and serum samples from the same donors as whole blood samples were also screened on Theranos Analyzer. The results showed that Lithium-Heparin plasma and serum should not be used for Theranos BNP test based on the results.

Table [SEQ Table * ARABIC]: Whole Blood Screen

Whole Blood Sample	Mean RLU	CV%	Mean Conc
F1	352	17	OORL
F2	404	26	OORL
F3	538	37	OORL
F4	277	16	OORL
F5	314	28	OORL

M1	579	37	OORL
M2	418	20	OORL
M3	381	9	OORL
M4	563	29	OORL
M5	364	28	OORL

Table [SEQ Table * ARABIC]: EDTA Plasma Screen

EDTA Plasma Sample	Mean RLU	CV%	Mean Conc
F1	452	26	OORL
F2	369	23	OORL
F3	447	33	OORL
F4	282	23	OORL
F5	459	36	OORL
M1	452	41	OORL
M2	531	38	OORL
M3	437	53	OORL
M4	380	66	OORL
M5	416	10	OORL

Table [SEQ Table * ARABIC]: Lithium-Heparin Plasma Screen

Li-Hep Plasma Sample	Mean RLU	CV%	Mean Conc	CV%
F1	822	72	OORL	
F2	465	30	OORL	
F3	497	42	OORL	
F4	545	20	OORL	
F5	385	51	OORL	
M1	674	35	OORL	
M2	1909	92	262	8
M3	2047	45	152	52
M4	1511	26	109	36
M5	2211	19	171	19

Table [SEQ Table * ARABIC]: Serum Samples Screen

Serum Sample	Mean RLU	CV%	Mean Conc	CV%
F1	640	24	OORL	
F2	745	25	44	33.199
F3	706	19	OORL	
F4	1243	60	115	54.561
F5	858	54	OORL	
M1	1260	33	84	50.074
M2	433	40	OORL	
M3	1382	29	96	43
M4	577	38	OORL	
M5	565	24	OORL	

2.16 Whole Blood Spike Recovery and Hematocrit Effect

The whole blood recovery was determined with whole blood spiked at eight levels. The spiked whole blood samples were measured on Theranos Analyzer, and the remaining spiked whole blood was centrifuged and took the plasma to test on Theranos Analyzer to evaluate hematocrit effect. Whole Blood is not recommended for Theranos BNP test since the sensitivity for whole blood samples was low. If whole blood has to be used for Theranos test, a new calibration curve in whole blood is needed to calculate BNP concentration. The spiked BNP was found to fully concentrate into the plasma prepared from the spiked whole blood. The recovery of BNP from plasma was 1.7 fold of that from whole blood samples.

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

Whole Blood	Signal, RLU		Back-Calculated Conc, pg/mL		
Spiked [BNP] (pg/mL) in Sample	Mean RLU	CV%	Mean Conc	CV%	% Recovery
2560	103762	29	2299	18	90
1280	19670	15	846	9	66
640	8995	13	521	9	81
320	3484	7	261	6	82
160	1653	10	125	12	78
80	699	23	OORL		
40	600	24	OORL		
0	479	26	OORL		

Plasma (From the Whole Blood)	Signal, RLU		Back-Calculated Conc, pg/mL		
Spiked [BNP] (pg/mL) in Sample	Mean RLU	CV%	Mean Conc	CV%	% Recovery
2560	220647	24	3882	18	152
1280	43592	15	1356	9	106
640	16025	16	747	10	117
320	6962	23	436	17	136
160	2588	12	201	12	125
80	977	18	54	37	68
40	590	3	OORL		
0	256	24	OORL		

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

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Hematocrit Effect

[SHAPE * MERGEFORMAT]

2.17 Interfering Matrices

Hemolyzed, icteric and lipemic EDTA plasma samples obtained from Bioreclamation were screened on Theranos Analyzer, BNP concentrations for all the samples are OORL. The recovery of BNP spiked into these potentially interfering matrices was evaluated on Theranos Analyzer. The results from spike recovery indicate that hemolyzed and lipemic EDTA plasma samples should not be used for Theranos BNP assay due to low recovery.

Table [SEQ Table * ARABIC]: Interfering Matrices Screen

Hemolyzed Plasma	Mean RLU	CV%	Mean Conc
H1	480	34	OORL
H2	551	37	OORL
H3	384	48	OORL
H4	476	51	OORL
H5	284	30	OORL

Lipemic Plasma	Mean RLU	CV%	Mean Conc
L1	478	47	OORL
L2	421	24	OORL
L3	363	28	OORL
L4	350	48	OORL
L5	297	23	OORL

Icteric Plasma	Mean RLU	CV%	Mean Conc
I1	425	24	OORL

I2	433	30	OORL
I3	347	32	OORL
I4	484	18	OORL
I5	508	38	OORL

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

A. Hemolyzed Plasma			Back-Calculated Conc, pg/mL		
Spiked [BNP] pg/mL	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**	**%Recovery**
2560	216434	15	2383	7	93
1280	48330	11	1066	7	83
640	15715	31	493	23	77
160	2095	29	90	28	57
80	1149	18	50	18	63
0	473	26	OORL		

B. Lipemic Plasma			Back-Calculated Conc, pg/mL		
Spiked [BNP] pg/mL	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**	**%Recovery**
2560	42511	15	983	9	38
1280	38315	18	919	11	72
640	11492	13	396	11	62
160	1659	13	72	13	45
80	1146	13	50	13	63
0	406	37	OORL		

C. Icteric Plasma			Back-Calculated Conc, pg/mL		
Spiked [BNP] pg/mL	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**	**%Recovery**
2560	179791	13	2187	6	85
1280	46964	11	1048	7	82
640	17435	10	527	6	82
160	2452	7	105	7	66
80	953	15	72	84	89

0	425	24	OORL		

Figure [SEQ Figure * ARABIC]: Spiked Recovery for Hemolyzed Plasma
[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Spike Recovery for Lipemic Plasma
[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Spiked Recovery for Icteric Plasma
[SHAPE * MERGEFORMAT]

2.18 Specificity

Five RF positive EDTA plasma samples and five HAMA positive EDTA plasma samples were tested for specificity of Theranos BNP Assay. The results showed that there was no false positive observed due to the presence of rheumatoid factor antibody or human anti-mouse antibody.

Table [SEQ Table * ARABIC]: RF Positive EDTA Plasma Samples

RF Positive Plasma Sample	Mean RLU	CV%	Mean Conc
RF#1	467	29	OORL
RF#2	546	27	OORL
RF#3	597	48	OORL
RF#4	494	29	OORL
RF#5	577	38	OORL

Table [SEQ Table * ARABIC]: HAMA Positive EDTA Plasma Samples

HAMA Positive Plasma Sample	Mean RLU	CV%	Mean Conc
HAMA#1	465	25	OORL
HAMA#2	342	35	OORL
HAMA#3	447	30	OORL
HAMA#4	511	36	OORL
HAMA#5	499	15	OORL

2.19 Stability Studies

Stability monitoring is ongoing for the the assay reagents stored at 4°C and protected from light.

3. REFERENCES

1. Rawlins ML, Owen WE, and Roberts WL. Performance Characteristics of Four Automated Natriuretic Peptide Assays. Am J Clin Pathol, 2005, 123:439-445