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Bone Alkaline Phosphatase Development and Validation Report 2010

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Assay Development Report
Bone Alkaline Phosphatase: Capture 12

A) Assay Development[XE "Assay Development"][XE "Assay Development"]

I) Assay Information[XE "Assay Information"]

1. Enzyme Background[XE "Analyte Background"]

Alkaline phosphatase is an enzyme that catalyzes the removal of phosphate groups by hydrolysis. In humans and most mammals, there are four isoenzymes present in the body: placental, placental-like, intestinal, and tissue non-specific. The tissue non-specific enzyme is abundant throughout the body but is represented by three different isoforms that are concentrated in the bone, kidney, and liver tissues respectively. The bone and liver isoforms of the enzyme account for the majority of circulating alkaline phosphatase (85-95%) and are generally present in equal amounts. Bone alkaline phosphatase (BAP exists as a tetrameric glycoprotein bound to the cell surface of osteoblasts but also circulates in the system as a dimer[1]. Osteoblasts are cells that play a role in bone formation and studies have shown that bone alkaline phosphatase levels can reflect the metabolic status of osteoblasts, making BAP levels a good marker for bone turnover. In recent years serum bone-AP levels have been used for assessing and monitoring the treatment of patients with conditions such as osteoporosis and Paget's disease of the bone. In osteoporosis there is loss of bone density and weakening of bone structure and in Paget's disease of the bone there is a disconnection between bone formation and resorption which generally leads to abnormal bone growths. BAP has been used to track changes in bone turnover over the course of antiresorptive and other therapies for bone growth disorders. Bone-AP is generally high in growing children and in people over 50 years old and is elevated in some growth and disorders such as acromegaly and hyperthyroidism.

REFERENCES

1. Price CP. Multiple forms of human serum alkaline phosphatase: detection and quantitation. *Ann.Clin.Biochem.* 1993; 30: 355- 372.

Bone Alkaline Phosphatase Range:

Normal

- Female (25 -44, Premenopausal): Range 11.6 – 29.6 U/L , Median 18.3 U/L_1
- Female (≥45 , Postmenopausal): Range 14.2 – 42.7 U/L , Median 25 U/L_1
- Male (≥25 , Postmenopausal): Range 15.0 – 41.3 U/L , Median 23.2 U/L_1

Disease

- Osteoporosis with Anti-Resorptive Therapy: ~4 – 17.9 U/L_1
- Osteoporosis No Anti-Resorptive Therapy: ~6 – 25.9 U/L_1
- Osteoporosis (drug status not clarified): Mean 29.8 U/L_2
- Osteomalacia: Mean 61.7 U/L_2
- Paget's Disease of Bone: Mean 199.6 U/L_2
- Hyperparathyroidism: Mean 29.3 U/L_2

REFERENCES

1. MicroVue BAP EIA Kit Insert, Ref 8012. Data obtained from attending clinical studies. Quidel Corporation.
2. Baltazar Gomez, Jr; Shiva Ardakani; Julia Ju et al; Monoclonal Assay for Measuring Bone-Specific Alkaline Phosphatase Activity in Serum.
Clin.Chem. 41/11, 1560-1566 (1995).

2. Reference Assays[XE "Reference Assays"]

Chosen Reference ELISA Kit
MicroVue BAP
Quidel
Catalog Number: 8012
Lot Number:

3. Assay Reagents[XE "Assay Reagents"]

A. Capture Antibody

Vendor	Lifespan
Catalog #	LS-C38190
Current Lot	22248
Stock Concentration	1mg/mL (un-conjugated)

Working Concentration	5ug/mL (NH2 conjugated to biotin)
Storage	2 – 8°C

B. Detection Antibody: N/A

C. Analytes

Vendor	US Biological
Catalog #	P4071-13
Current Lot	L3100960
Stock Concentration	1mg lyophilized powder at 0.5U/mg Activity
Working Concentration	Reconstitute to 1000U/L using DI water.
Storage	-20°C

Vendor	CalZyme
Catalog #	124A0001
Current Lot	4-1-8
Stock Concentration	Sold by units of activity, lyophilized powder at 0.5U/mg activity
Working Concentration	Reconstitute to 10,000U/L using DI water.
Storage	-20°C

D. Sample Diluent

Composition	3% BSA +0.05M TBS + 0.05% NaN3 + 1.5mg/mL EDTA
Storage	2 -8 C

4. Reagent Handling and Storage (Analyte)[XE "Reagent Handling and Storage (Analyte)"]

Special instructions for handling & storage of analyte:

- **Reconstitution**
  1. Analyte comes as a white powder.
  2. Allow analyte tube to come to room temperature briefly.
  3. Centrifuge vial at 2,000 rpm for 1 minute to let powder settle.
  4. Open vial and add enough de-ionized water to bring analyte to 1000U/L nominal activity for US Biological stock or to 10,000 U/L for CalZyme stock. For example, if analyte came as 1mg at 0.5U/mg activity, adding 500uL of water would give the desired activity level of 1000U/L.
  5. After adding water, seal vial, vortex briefly, and then place on rotisserie to mix for approximately 1-5 minutes.
  6. Once rotisserie mixing is done, centrifuge vials again as in step 3.
  7. Once all vials being reconstituted have been centrifuged, open them and pool them into a container.
  8. Mix solution, aliquot into tubes for later use, label tubes and store at -20°C.
- **Analyte Storage**
  Dry powder is stored at -20°C. Reconstituted bone AP solutions in water are generally used as in the house stock and are stored at -20°C. For final activity assignment, analyte can be tested on the MicroVue BAP Kit.

5. Protocols[XE "Protocols"]

Recommended Theranos reader protocols:
Protocol features: 10/10/10 assay protocol with 10X sample dilution, 6x post detection antibody washes with 1 minute incubations, 2x pre wash. The assay uses the bone alkaline phosphatase in sample as the detection so no detection is loaded into cartridges.

	Protocol name	Svn #	Sample dilution	Well positions
1	Generic_10	2890	10x	Any of 1-6

II) Assay Optimization[XE "Assay Optimization"]

6. Antibody Screening Summary[XE "Antibody Screening Set Summary"]

In the first round of screening for the bone AP assay, candidates 1 through 8 were biotinylated using both SH and NH2 chemistries and tested on micro-titer plates to look for the best candidate capture. Since the analyte itself is an enzyme, the assay was

performed by incubating different levels of the analyte on the capture surface, washing, exposing the surface to the appropriate substrate, and using the generated chemiluminescence. As a general rule the NH2 conjugates for a given antibody tended to give better results in terms of higher top/bottom signal and lower CVs. From the first MTP testing, captures 3, 4, and 6 were the top candidates and moved to Theranos system testing. On the Theranos system capture #4 was the strongest candidate by far and development continued using that capture with no detection. However, near the end of development testing showed that clinical correlation was not strong enough so some of the original antibodies were tested conjugated to AP as detections to see if results improved and new antibodies were ordered for screening. The use of detection conjugates failed to improve results so that approach was dropped and the capture only assay was further pursued. After various rounds of testing, several new antibodies emerged as strong candidates based on good results during screening and preliminary testing of clinical correlation with those antibodies. The strongest second round candidates were c-abs 12, 13, 14, and 16. Of those, the choice was made to restart development using captures 12 and 16 in parallel, with the goal of having a ready back up already validated should that be needed.

Bone Alkaline Phosphatase Antibody Key

Code	Vendor	Catalog #	Clone	Type
1	Abcam	AB17272	O.G.2	Mouse Monoclonal; IgG
2	Abcam	ab68716	N/A	Sheep Polyclonal; IgG
3	Novus	NB100-66384	BGN/03/661	Mouse Monoclonal; IgG
4	Santa Cruz Biotech	sc-81754	B4-78	Mouse Monoclonal; IgG
5	Thermo Scientific	MA1-82961	BGN/03/66KF44	Mouse Monoclonal; IgG
6	Thermo Scientific	MA1-82963	BGN/03/662	Mouse Monoclonal; IgG
7	Thermo Scientific	MA1-82959	BGN/03/66KF41	Mouse Monoclonal; IgG
8	Thermo Scientific	MA1-82960	BGN/03/66KF42	Mouse Monoclonal; IgG
9	US Biological	P4071-4H	10B140	Mouse Monoclonal; IgG
10	US Biological	P4071-4F	10B138	Mouse Monoclonal; IgG
11	Lifespan Biosciences	LS-C17269	Un-specified	Mouse Monoclonal; IgG
12	Lifespan Biosciences	LS-C38190	Un-specified	Mouse Monoclonal; IgG
13	Millipore	MAB4349	clone TRA-2-49/6E	Mouse Monoclonal; IgG

14	Millipore	MAB4354	clonc TRA-2-54/2J	Mouse Monoclonal; IgG
15	Novus	H00000249-M01	4H1	Mouse Monoclonal; IgG
16	Novus	H00000249-D01P	N/A	Rabbit Polyclonal; IgG
17	US Biological	P4071-04E	10B141	Mouse Monoclonal; IgG
18	US Biological	P4071-11B	N/A	Sheep Polyclonal; IgG

Antibody Screening Summary

	No D-Ab	Dab #2	Dab #5	Dab #7	Dab #8	Dab #9	Dab #10
Cab #1							
Cab #2							
Cab #3							
Cab #4							
Cab #5							
Cab #6							
Cab #7							
Cab #8							
Cab #9							
Cab #10							
Cab #11							
Cab #12							
Cab #13							
Cab #14							
Cab #15							
Cab #16							
Cab #17							
Cab #18							

Number of Capture antibodies tested: 18
Number of Detection antibodies tested: 6
Total Number of antibody configurations tested: 31

No Response
Poor response but potential
Modulation but background or other problem
Modulation, good candidate pair

7. Cross Reactivity and Interference[XE "Cross Reactivity and Interference"]

Potential cross reactivity to the major human alkaline phosphatase enzymes of interest was tested on bone AP capture candidates 12 and 16 by running 6 point curves of the liver, intestinal, and placental alkaline phosphatase enzymes in assay buffer on the Theranos bone AP assay using surfaces coated with those captures/ Control curves with bone AP were also run. Testing was done using a 10-10 protocol. The potential cross reactants were tested at the following levels: liver AP in the range from 1.95 to 2,000 U/L, intestinal AP in the range from 4.88 to 5,000 U/L, and placental AP in the range from 3.2 to 10,000 U/L. TRAP5b was also tested for potential cross reactivity because it is another phosphatase enzyme that is slated to be on the same test as the bone AP assay. TRAP5b was tested in the range from 1.95 to 15.9 U/L. Although the cross reactivity for both 12 and 16 to liver and intestinal AP looks high, it is still equal to or less than that seen on the chosen reference kit by Quidel. When purified enzymes from CalZyme were tested in house on the Quidel kit, the Quidel kit has showed cross reactivity from 32 – 34% for the liver AP and cross reactivity of 11% for the intestinal AP.

Cross Reactivity Testing Summary Table

Bone AP C-Ab Conjugate=>	#12	#16
Avg % Reactivity to Liver AP	32.5	25.5
Avg % Reactivity to Intestinal AP	7.7	8.7
Avg % Reactivity to Placental AP	0.2	1.6
Avg % Reactivity to TRAP5b	0.0	0.0

Next came testing for potential interference of various substances on the performance of the Theranos bone AP assay. For interference testing, a given level of the substance on test was spiked into a six point set of bone AP calibrators in assay buffer and the recovery of the bone AP in those solutions was measured against that in a control made using the same diluent and bone AP stock but without any of the other substances on test. Due to structural and functional similarity, liver AP, intestinal AP, placental AP, and TRAP5b were tested. The data is summarized below along with test levels. Intestinal AP at 1,250 U/L consistently lowered recovery to 66% for the bone AP curve. The liver AP at 400 U/L led to inconsistent bone AP recovery with the top two points being lower than expected and the three lowest points being acceptable. The end conclusion appears to be that the tested levels of both liver and intestinal AP would be expected to interfere with assay results.

Interference Testing Summary Table

Substance Tested	Level Tested	Avg %Recovery of BAP	Interference?
Placental AP	2000 U/L	98	No

TRAP 5b	31.8 U/L	100	No
Liver Alkaline Phosphatase	400 U/L	86	Yes
Intestinal Alk Phos	1250 U/L	66	Yes

8. Theranos Screen

The two current best capture antibodies from micro-titer plate screening - numbers 12 and 16 - were also tested on the Theranos system with bone AP solutions in assay buffer, blood, and plasma on a 10-10-10 protocol. Six point curves in each of the matrices were prepared in the range from 196 – 6.1 U/L with a zero also tested. For assay buffer calibrators, captures 12 and 16 gave similar modulation even though the actual RLUs were much higher for capture 12 compared to 16. Due to higher counts for that capture, #12 has higher S/B than #16. Also, for all matrices tested, c-ab #12 gave slightly lower signal and concentration CVs. For plasma, 12 and 16 both give ~72% recovery on average and again have similar modulation. For an unclear reason, capture 16 showed 92% average recovery in the whole blood and capture 12 gave 75%. The belief is that this is likely to be a fluke and that as with capture 12, the recovery of bone AP in whole blood and plasma is expected to be similar on capture 16. The overall testing reveals that both captures 12 and 16 give a good response in assay buffer, whole blood, and plasma and that although recovery of BAP is low in at least plasma for both captures, the results are consistent along the relevant range and should be something that can be calibrated for.

Summary of Results in Assay Buffer

Conjugate=>	**12**	**16**
Top/Bottom Signal	19805	1325
Std 5/6	817.23	41.08
Average Ratio (Cal 1-4)	2.35	2.60
Avg % Signal CV	10.0	13.0
Avg % Concentration CV	10.7	14.5

Summary of Results in Blood

Conjugate=>	**12**	**16**
Top/Bottom Signal	16	19
Std 5/6	4.16	4.57
Average Ratio (Cal 1-4)	1.41	1.44
Avg % Signal CV	9.1	13.5

Avg % Concentration CV	9.1	14.8

Summary of Results in Plasma

Conjugate=>	**12**	**16**
Top/Bottom Signal	9	9
Std 5/6	2.18	2.56
Average Ratio (Cal 1-4)	1.41	1.40
Avg % Signal CV	10.4	14.0
Avg % Concentration CV	9.6	16.2

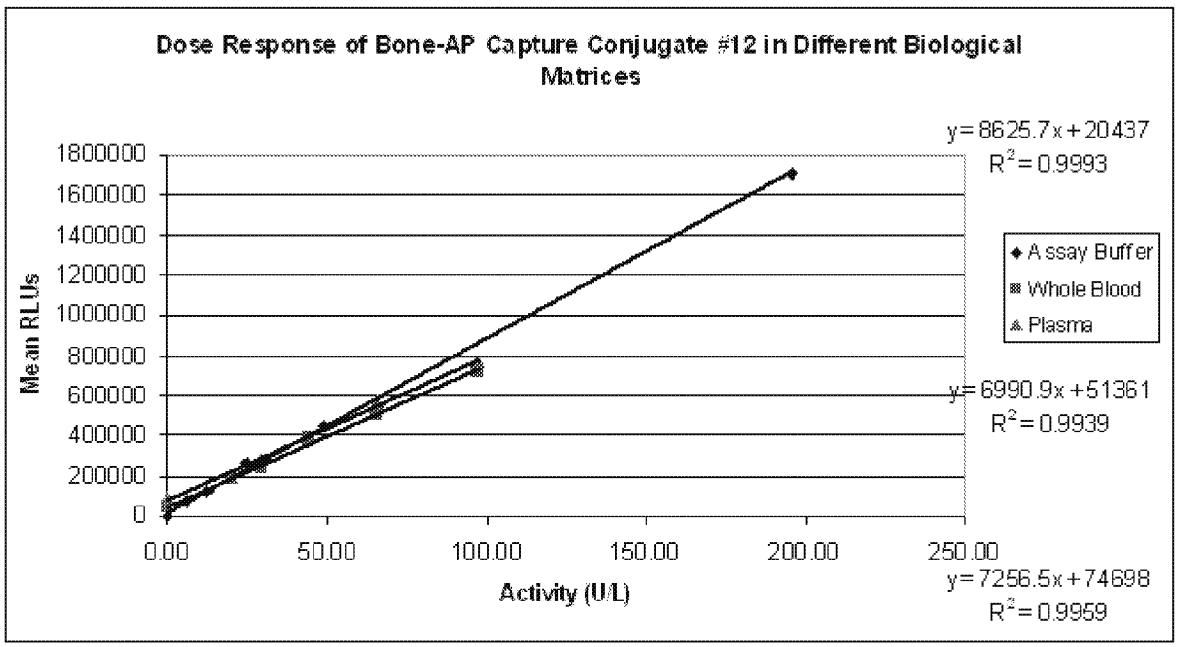
Summary of % Recovery of Bone AP in Whole Blood

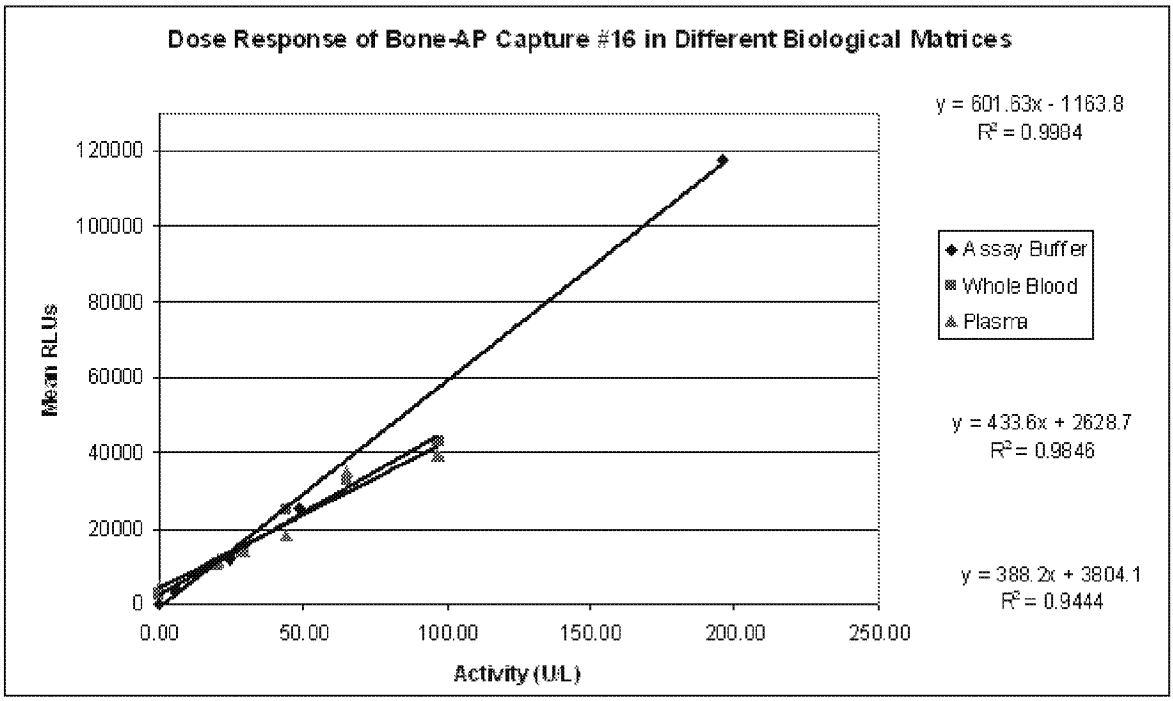
Spike Level	C-Ab 12	C-Ab 16
Activity (U/L) in Sample	% Recovery	% Recovery
97.20	76	85
65.26	75	97
44.15	83	108
29.43	68	84
19.94	72	87
0.00	N/A	N/A

Summary of % Recovery of Bone AP in Plasma

Spike Level	C-Ab 12	C-Ab 16
Activity (U/L) in Sample	% Recovery	% Recovery
97.20	78	73
65.26	80	**99**

4.15	74	72
29.43	68	72
19.94	57	75
0.00	N/A	N/A





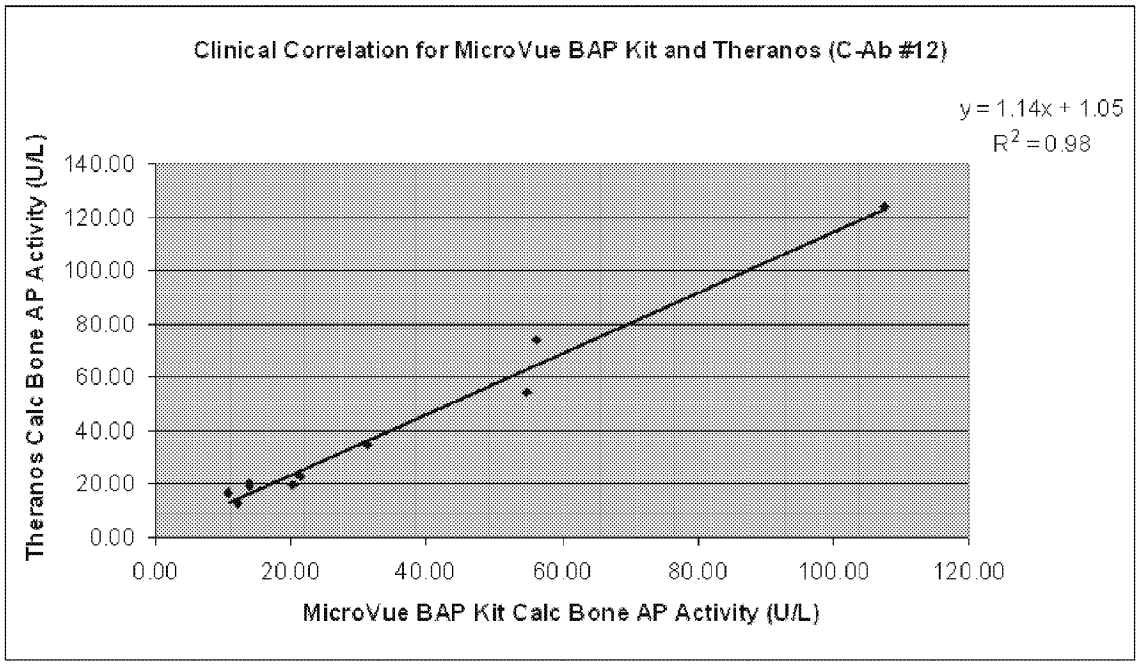
9. Training Set

To confirm that the chosen bone AP capture antibody and the Theranos bone AP assay were of sufficient quality, ten clinical serum samples were tested on both the Theranos system and the MicroVue BAP commercial kit to look at correlation. The clinical samples came from an alkaline phosphatase panel and from osteoporosis and renal failure patients. The correlation equation for the clinical sample calculated bone AP levels on the Theranos system and the MicroVue BAP kit was: y = 1.14x + 1.05, R2 = 0.98. The results showed sufficiently strong clinical correlation and that the chosen antibody was able to bone AP in real human samples.

Summary Table of Clinical Results

Sample	Theranos Calculated Activity (U/L)	MicroVue BAP Kit Calc Activity (U/L)	% (Theranos/MicroVue)
37	17.11	10.61	161
41	20.00	13.72	146
43	23.00	21.30	108
45	19.03	13.72	139
46	12.79	12.12	106
49	19.56	20.14	97
50	34.82	31.25	111

51	54.34	54.85	99
53	124.16	107.56	115
54	74.09	56.17	132



10. Whole Blood and Plasma Screen[XE "Whole Blood and Plasma Screen"]

Blood (potassium EDTA anti-coagulated) was obtained from a blood bank and blood and plasma (obtained by centrifugation) were analyzed in the Theranos System. Testing was done using sample diluent with 1.5mg/mL EDTA added (for both calibration and sample testing). For matched patients, the results in the plasma came out higher than those in the whole blood, demonstrating a hematocrit effect for bone alkaline phosphatase. Averaging across all matched patients tested here, the calculated BAP in plasma was about 1.78X the level calculated in the whole blood.

Patient Info		Whole Blood [Calc.] (U/L)	Plasma [Calc.] (U/L)	% (Plasma Calc Conc/ Blood Calc Conc)
Date	Tube #			
8/18/2010	1	15.97	28.08	176
8/18/2010	2	12.25	25.90	211
8/18/2010	3	13.11	23.39	178
8/19/2010	4	14.11	29.74	211

8/19/2010	6	11.43	16.64	146
8/19/2010	7	15.27	30.28	198
8/19/2010	8	11.04	14.65	133
8/20/2010	9	12.87	19.24	149
8/20/2010	10	23.95	37.94	158
	Mean BAP (U/L)	14.38	25.68	

11. Capture Antibody Titration[XE "Capture Titration, Protocol Comparison, and Surface Optimization"]

For capture titration, bone AP assay response was tested on the Theranos system for surfaces coated with 4 different concentrations of the chosen biotinylated capture conjugate. A six point curve in assay buffer was tested in the range from 196 - 0U/L and whole blood was tested with 119.28, 59.64, and 0 U/L spikes. The capture antibody seemed to saturate at around 5ug/mL, with the counts for 5, 10, and 20 ug/mL of capture looking very similar. Since going higher than 5ug/mL did not yield any significant improvement in results and the antibody coated at that level gave good CVs and modulation in both blood and assay buffer, the chosen capture concentration for bone AP c-ab #12 is 5ug/mL.

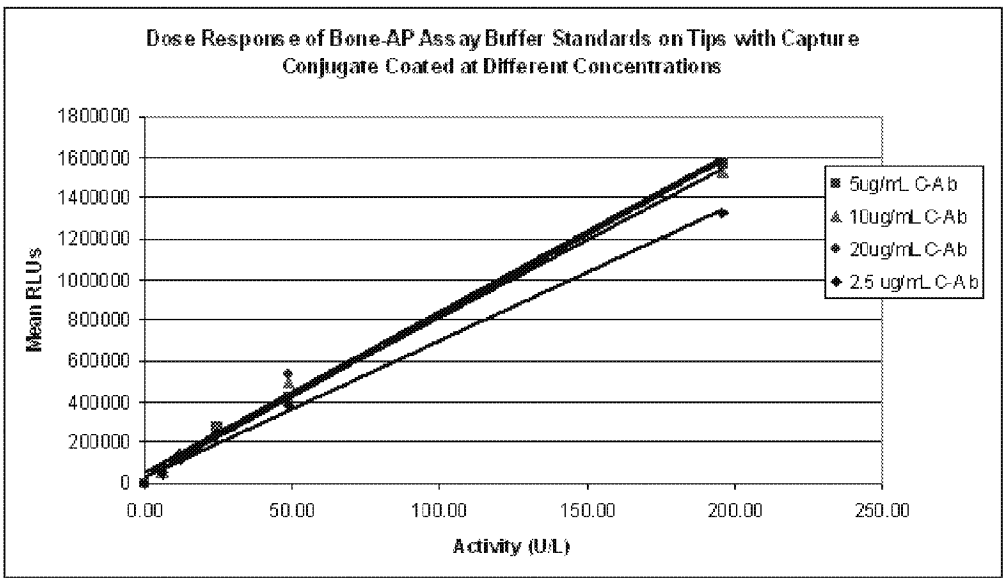
Summary of Assay Buffer Results

Capture Concentration (ug/mL) =>	2.5	5	10	20
Std 1/6	10635	11987	10186	11058
Std 5/6	391.72	506.24	383.16	551.63
Average Ratio	80.25	103.12	78.49	112.06
Avg % Signal CV	11.8	6.9	12.2	7.6
Avg % Conc CV	12.67	7.47	10.15	5.18
Avg% Recovery	101	100	98	101

Summary of Whole Blood Results

Capture Concentration (ug/mL) =>	2.5	5	10	20

Std 1/3	26	35	24	22
Std 3/4	14.17	20.01	10.09	11.42
Average Ratio	8.00	10.87	6.25	6.66
Avg % Signal CV	6.88	6.09	8.93	10.17
Avg % Conc CV	7.40	6.74	9.25	8.99
Avg% Recovery	73	70	58	63



12. Detection Titration

Because the analyte itself is an alkaline phosphatase enzyme, the assay was initial tested without detection using direct analysis of the bound analyte from sample. However, some detection conjugate screening was done just to make sure that the assay would not benefit from being run in the more traditional way with a detection antibody.

13. Buffer Testing

Bone AP capture #12 was tested coated at 5ug/mL in assay buffer, sea block, or starting block to see if one blocker was superior to another. A six point curve in assay buffer in the range from 196 – 0 U/L and whole blood at spike levels of 106.2 and 0 U/L were tested on tips coated with the different blockers. The performance of the Theranos bone AP assay using c-ab #12 was similar in all three blockers for both blood and assay buffer, suggesting

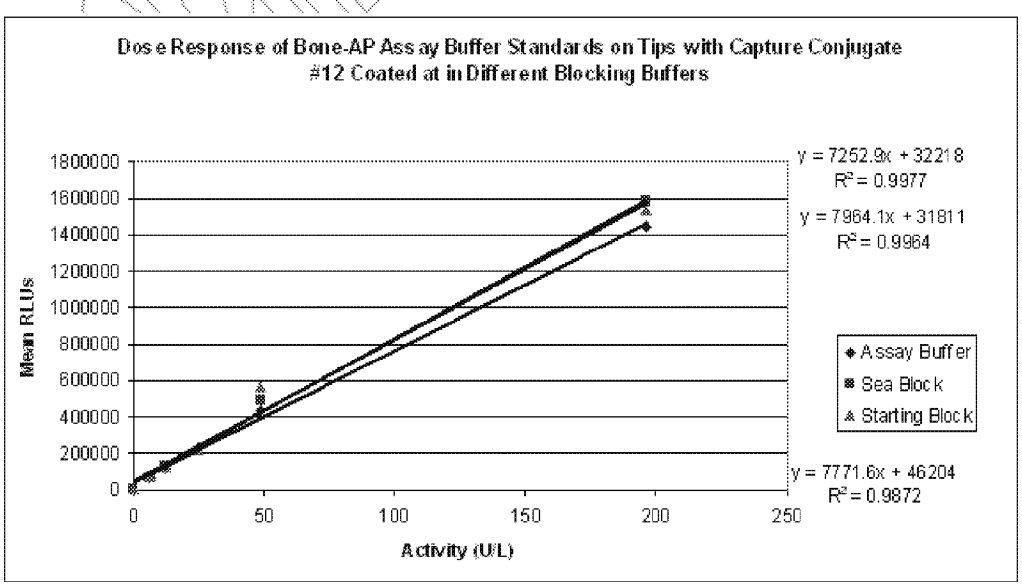
that there was no advantage to one blocker over another for the assay. As such, the chosen capture diluent/blocker for the bone AP assay with c-ab #12 is assay buffer (3% BSA + 0.05% NaN3 in TBS).

Assay Buffer Calibrator Result Summary

Capture Blocker=>	Assay Buffer	Sea Block	Starting Block
Std 1/6	11029	9010	13462
Std 5/6	521.22	380.15	647.96
Average Ratio	106.02	77.86	131.34
Avg % Signal CV	11.9	12.2	10.3
Avg % Conc CV	13.75	12.49	11.95
Avg% Recovery	100	100	101

Blood Testing Result Summary

Capture Blocker=>	Assay Buffer	Sea Block	Starting Block
Std 1/2	21	22	22
Average Ratio	20.63	21.85	22.38
Avg % Signal CV	11.47	12.44	9.95
Avg % Conc CV	12.71	13.50	10.51
Avg% Recovery	44	43	43



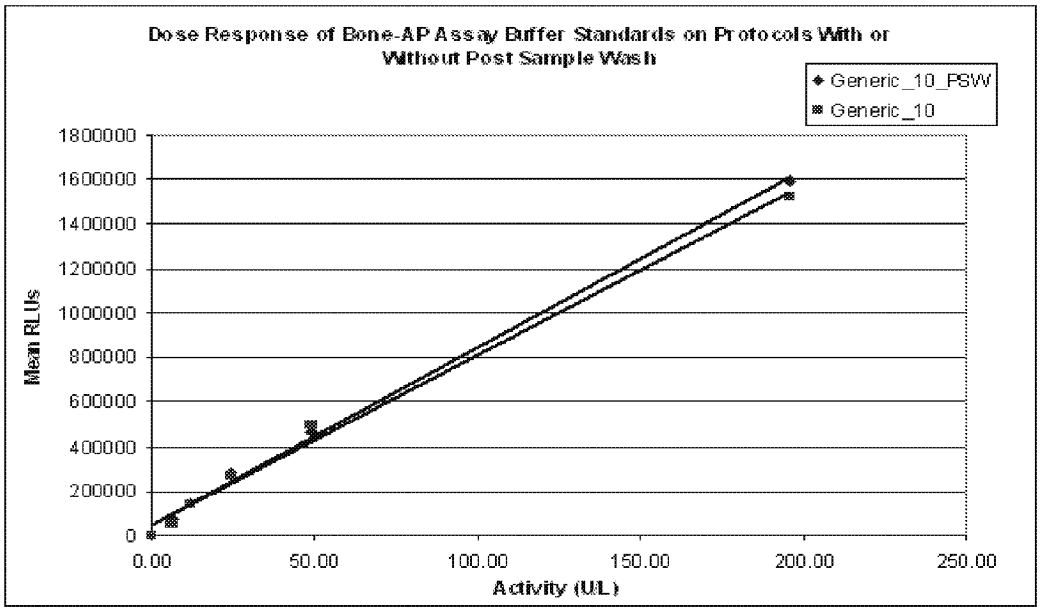
[XE "Buffer Effects\: Testing Different Sample Diluents"]Bone alkaline phosphatase is an enzyme that requires metal ions to function properly. As such, the enzyme is sensitive to chelators such as EDTA. However, EDTA is one of the standard anti-coagulants and the current chosen anti-coagulant for the Theranos blood transfer device. Because of this, and because the blood that is generally used for development testing in house contains EDTA, experiments have been done to see if adding EDTA to the sample diluent helps correct for the loss of bone AP activity in blood samples with EDTA and samples exposed to the EDTA in the Theranos blood transfer device (BTD). In the first round of testing it was found that testing samples exposed to the BTD or blood or plasma samples containing EDTA on bone AP cartridges where there was no EDTA in the sample diluent during calibration or testing, tended to give low recovery. When 1.5mg/mL of EDTA was added to the sample diluent (the same level used for BD vaccutainers with EDTA), the recovery came up to a more reasonable level. Based on overall testing, the current Theranos bone AP assay is designed to work with samples that contain EDTA and thus the assay sample diluent contains EDTA.

14. Edison Protocol Optimization

Two versions of the same basic Edison protocol were tested for the bone AP assay: one with a post sample wash and one without. Six point calibrator curves in triplicate (2 tips per cartridge, 3 cartridges per solution) were tested on the two 10X diluting 10-10-10 protocols, one with a post sample wash and one without. The addition of a post sample did not seem to have significant positive or negative effects on assay response for bone alkaline phosphatase assay using capture 12 so the chosen protocol for bone AP on this capture is 10-10-10 timing, 10X sample dilution, and no post sample wash.

Summary of Assay Buffer Results

Protocol=>	Generic_10_PSW	Generic_10
Std 1/6	11680	10186
Std 5/6	559.98	383.16
Average Ratio	113.78	78.49
Avg % Signal CV	11.4	12.2
Avg % Conc CV	12.72	10.15
Avg% Recovery	100	98



15. Precision Tests[XE "Precision and CV Test"]

Formal precision testing was not completed for this assay but data for the standard curve on two different lots of tips was compared to give an idea of precision. Although some points gave CVs of higher than 10%, the average inter-and intra-lot CVs were less than 10% and the highest CVs were less than 15%, which is the FDA cutoff. Results show that the assay should be reasonably precise.

Mean Inter-Run % Signal CV=8.7
Mean Intra-Run % Signal CV=7.4
Mean Inter-Run % Concentration CV=9.1
Mean Intra-Run % Concentration CV=7.8

Summary Data

Activity (U/L) in Sample	Lot 1 % Signal CV	Lot 2 % Signal CV	Combined Lot Analysis % Signal CV	Lot 1 % Conc CV	Lot 2 % Conc CV
196.00	8.4	2.9	8.4	8.9	3.0
49.00	7.6	9.9	7.6	8.0	10.4
24.50	13.9	8.7	13.9	14.6	9.1
12.25	12.3	4.0	12.3	12.9	4.2
6.13	5.8	6.2	5.8	6.1	6.6
0.01	4.0	4.9	4.0	4.2	5.1

For CV testing, one bone alkaline phosphatase assay buffer solution in the mid range of the assay (51.12 U/L) was assayed across 38 different instruments using tips from 24 different trays to determine the mid-range coefficient of variation (CV).

Summary

Total Signal CV (any cartridge, any instrument): 11.9%
Total Activity CV (any cartridge, any instrument): 13.2%
Average Intra-Cartridge Signal CV: 8.8 %

Average Intra-Cartridge Activity CV: 9.8 %
Average Inter-Cartridge Signal CV: 8.2%
Average Inter-Cartridge Activity CV: 9.1%

Summary of Total Concentration % CV

Mean Calculated Activity (U/L)	STDev	% Activity CV
45	6	13.2

16. Calibrator Comparison[XE "Calibrator Comparison"]

To confirm that the candidate bone AP analyte stocks for Theranos can be detected on the chosen reference commercial kit and determine their relative activity, dilutions of these analyte stocks were tested on the MicroVue BAP kit. To look at the cross reactivity of the commercial kit to the liver and intestinal placental forms of alkaline phosphatase, those reagents were also tested. To confirm that the Theranos bone AP assay was able to see the reference kit calibrators, those reagents were tested on the Theranos bone AP assay and their recovery calculated. The MicroVue kit was able to see both of the candidate bone AP stocks for Theranos, with the Calzyme reagent showing lower recovery relative to nominal and the US Biological stock showing higher recovery. Interestingly, the commercial kit showed higher % cross reactivity to the liver and intestinal forms of AP than their insert claims, however, when their original testing was done there were no pure sources of these reagents to test so they had to use heat inactivation and other methods to isolate the specific forms of alkaline phosphatase, which could be the source of the discrepancies. The kit claims 3 - 8 % cross reactivity to the liver form and 0.4% to the intestinal. The calculated cross reactivities for the pure materials on the kit were 32% for liver AP and 11% for intestinal AP.

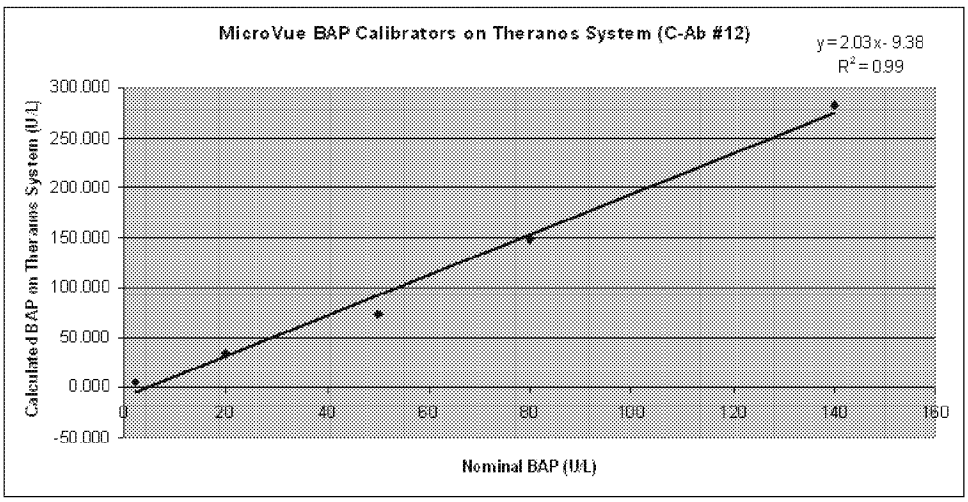
Summary Table of Percent Recovery for Reagents Tested on MicroVue BAP Kit

Analyte	Avg % Recovery on Kit
US Biological Bone AP (50-12.5U/L)	158
Calzyme Bone AP (50-12.5U/L)	71
Calzyme Liver AP (500U/L)	32
Calzyme Intestinal AP (1,250U/L)	11

The MicroVue BAP kit calibrators were also tested on the Theranos bone AP assay to make sure that our system sees the calibrators for the reference kit. The kit calibrators gave fairly linear recovery on the Theranos system but the recovery was high, around 176% on average looking at the most consistent points. The only suspected reason for the high recovery is the fact that the kit calibrators contain a diluent with extra zinc and magnesium spiked in, which might make them more resistant to the chelating effects of the EDTA in the Theranos sample diluent, making them look more active relative to the Theranos calibrators which are not in a diluent spiked with extra zinc and magnesium. The good correlation for the clinical samples helps support this and suggests that the amount of extra zinc and magnesium spiked into the MicroVue calibrators may

be greater even that physiological levels in the average human, as the Theranos system and the kit agree very closely on most of the 20 samples tested for the extended clinical correlation.

Nominal BAP Activity (U/L) in Sample	Mean Calc Act (U/L)	Conc CV%	% Recovery
140	283.086	8.0	202
80	148.022	4.4	185
50	73.267	9.1	147
20	34.091	6.9	170
2	6.344	4.0	317
0	0.095	3.5	N/A



17. Dilution Linearity[XE "Dilution Linearity"]

Two clinical serum samples- one high and one low – were mixed together to test for dilution linearity. The nominal concentrations of the undiluted samples were set at the Theranos system reported concentration and those values were used to calculate the expected concentrations for the dilutions. The concentrations of the serial dilutions were calculated based on the volume ratios of the low and high sample used to create them and the nominal concentrations of the low and high samples. This testing was done for two sample pairs. Both sample pairs showed good dilution linearity for bone AP with the exception of one point in the first pair, which gave 79% recovery, but that is just 1% below the limit and all the dilutions below that gave recovery to within 20% of the target.

The following equation was used to determine the recovery percentage: 100*(calculated concentration/expected concentration). Except for the neat high and low samples which were set at 100% recovery by definition.

Summary of Results for Sample Pair 1

Nominal [BAP] U/L	Dilution from Neat High Sample	Calculated [BAP] U/L	% Concentration CV for Four Tips	% Recovery
94.03	1X (Neat high sample)	94.03	6.0	[100]
52.48	2X	41.60	12.2	79
31.70	4X	30.26	8.6	95
21.31	8X	24.45	12.1	115
16.12	16X	15.01	8.1	93
13.52	32X	14.23	4.7	105
12.22	64X	11.65	3.4	95
10.92	1X (Neat low sample)	10.92	7.5	[100]
			Average % Recovery	97

Summary of Results for Sample Pair 2

Nominal [BAP] U/L	Dilution from Neat High Sample	Calculated [BAP] U/L	% Concentration CV for Four Tips	% Recovery
81.24	1X (Neat Sample #53)	81.24	9.4	[100]
43.17	2X	42.96	6.3	100
24.14	4X	21.79	9.7	90
14.62	8X	14.75	12.7	101
9.86	16X	10.16	4.7	103
7.48	32X	7.66	10.2	102
6.29	64X	6.86	3.3	109
5.10	1X (Neat bulk serum 1)	5.10	5.5	[100]
			Average % Recovery	99

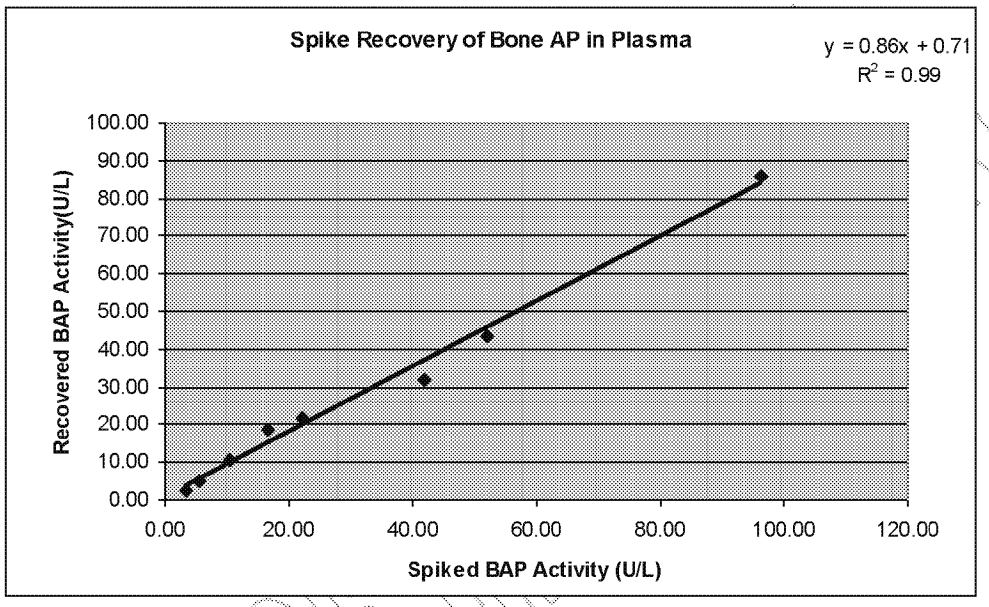
18. Recovery in Whole Blood and Plasma and Hematocrit Effect[XE "Recovery in Whole Blood and Plasma and Hematocrit Effect"]

To test spike recovery of bone alkaline phosphatase in whole blood and plasma, 7 point curves of bone AP were spiked directly into whole blood or plasma containing EDTA as the anti-coagulant. Zeros of the un-spiked blood and plasma were also tested to calculate the endogenous bone AP levels for the tested samples. Spiked solutions were then tested on the Theranos system using standard assay buffer as the diluent and recovery was calculated against an assay buffer calibration curve. For both directly spiked blood and plasma, recovery was fairly linear and generally to within 20% of target.

Directly Spiked Plasma Data

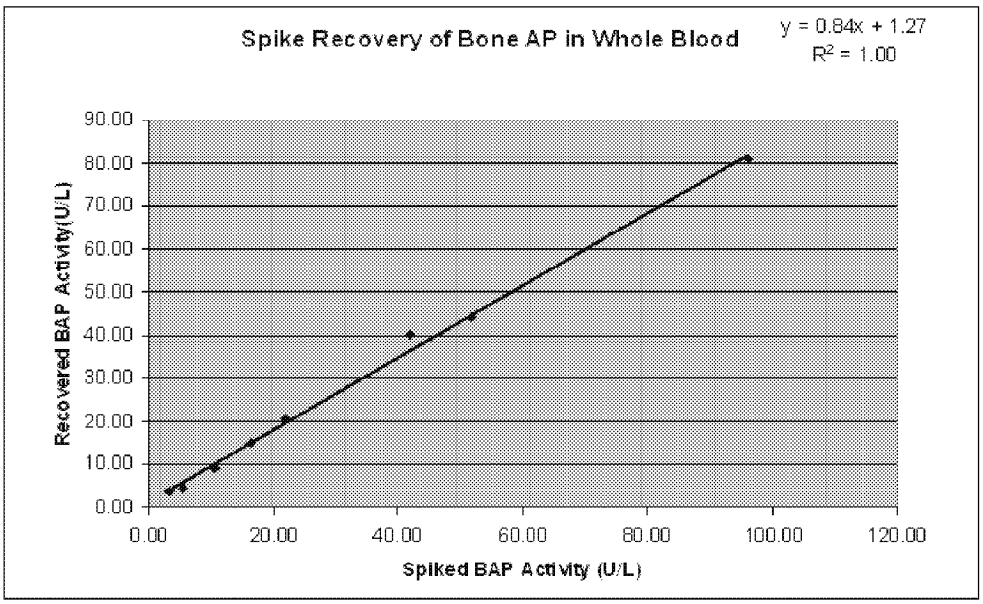
Spiked [BAP] U/L in sample	Recovered [BAP] U/L	Activity % CV	% Recovery
96.24	85.97	9.3	89
51.92	43.32	5.9	83
41.90	32.04	9.0	76
22.17	21.90	10.8	99
16.52	18.45	11.2	112

10.57	10.67	7.6	101
5.52	4.86	11.5	88
3.41	2.73	6.4	80



Directly Spiked Whole Blood Data

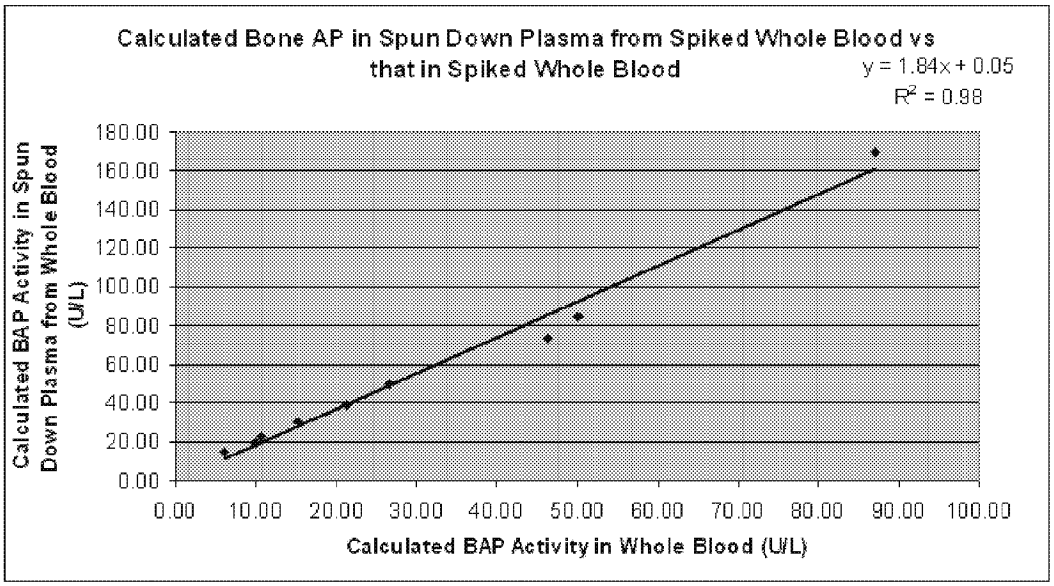
Spiked [BAP] U/L in sample	Recovered [BAP] U/L	Activity % CV	% Recovery
96.24	81.00	5.2	84
51.92	43.95	7.7	85
41.90	40.22	8.4	96
22.17	20.64	5.9	93
16.52	15.16	14.4	92
10.57	9.15	11.3	87
5.52	4.68	5.1	85
3.41	3.92	12.8	115



To look at hematocrit effect, the recovery of bone AP in spiked whole blood and in the plasma from the same spiked whole blood was compared. The spun down plasma from the spiked whole blood revealed a hematocrit effect and showed calculated BAP at roughly 1.5X the amount seen in the directly spiked whole blood.

Spun Down Plasma from Spiked Whole Blood

Spiked [BAP] U/L in sample	Recovered [BAP] U/L	Activity % CV	% Recovery
96.24	81.00	5.20	162
51.92	43.95	7.70	135
41.90	40.22	8.39	142
22.17	20.64	5.93	158
16.52	15.16	14.41	149
10.57	9.15	11.26	152
5.52	4.68	5.07	153
3.41	5.31	4.3	155



19. ULOQ and LLOQ[XE "ULOQ and LLOQ"]

ULOQ and LLOQ were calculated from plasma spike recovery data. The calculated ULOQ was 96.24 U/L and the calculated LLOQ was 3.41 U/L.

Spike Level		
Activity (U/L) in Sample	% Recovery	% Conc CV
96.24	89	9.3
3.41	80	6.4

20. Selectivity

For selectivity testing, bone AP was spiked into EDTA whole blood at 3 levels over endogenous for 5 males and 5 females, with spike levels of 92.36, 39.98, and 21.57 U/L. The spiked solutions and zeros of each patient's blood were tested on the Theranos system using sample diluent containing EDTA. Control assay buffer dilutions of the 10X concentrated assay buffer spike stocks were also tested. Recovery of bone AP was calculated against an assay buffer calibration curve (separate from the tested assay buffer dilutions of the spike stocks) with recovered activity defined as calculated minus endogenous. Results were fairly consistent across patients and spike levels, with recovery within 20% of 100%. However, for an unknown reason, patient 9 gave only 72% recovery at the second spike level and patient 10 gave only 64% recovery for the top spike level.

Summary of Overall Variability in BAP Recovery in Whole Blood Across 10 Patients

Activity (U/L) Spiked	Inter-Patient Mean % Recovery	STDev	Inter-Patient % CV
92.36	89	12.9	14.6
39.98	96	12.9	13.4
21.57	100	8.0	8.0

Summary Table of % BAP Recovery in EDTA Whole Blood for 5 Male Patients

Patient=>	1, Male	2, Male	3, Male	4, Male	5, Male
Activity (U/L) Spiked	%Recovery	%Recovery	%Recovery	%Recovery	%Recovery
92.36	84	81	86	80	99
39.98	111	105	88	86	101
21.57	107	95	110	97	101
Avg% Recovery	101	94	95	87	100

Summary Table of % BAP Recovery in EDTA Whole Blood for 5 Female Patients

Patient=>	6, Female	7,Female	8, Female	9, Female	10, Female
Activity (U/L) Spiked	%Recovery	%Recovery	%Recovery	%Recovery	%Recovery
92.36	104	88	96	107	64
39.98	95	89	100	72	115
21.57	106	99	82	103	96
Avg% Recovery	102	92	93	94	92

21. Capture Surface Stability[XE "Capture Stability"]

For capture surface stability, coated tips were calibrated using frozen assay buffer calibrators and then the tips were pouched with desiccant and stored at 4°C and room temperature. The test schedule for the tips wasT = 0 (calibration), 1, 2, 4, 8, 12, and 24 weeks. At each time point, four calibrators crossing the analyte range are tested on both the tips stored at RT and those stored at 4°C, with 4 tips per storage condition per calibrator tested at each test point. The wet test reagents for stability are those used for calibration of the tips and were set aside for the duration of the experiment and stored at 4°C. Tips were coated on the TomTec using 30-30-10 timing, 20ug/mL Ultravidin in carb-bicarb buffer, and 5ug/mL of capture 12 in assay buffer. The test protocol used was Generic10_svn2089. Stability was completed on March 1, 2011. The coated tips proved stable for 24 weeks at both 4C and RT.

Summary Table of % Recovery Relative to Nominal For Bone AP Test Tips Stored at 4°C or RT

Storage Temp	[BAP] U/L	T=0 % Recovery	T= 1 Week % Recovery	T=2 Weeks % Recovery	T=4 Weeks % Recovery	T=8 Weeks % Recovery	T=12 Weeks % Recovery	T=24 Weeks % Recovery
4C	197.380	100	109	99	85	93	89	89
	101.761	100	80	78	71	94	85	82
	43.270	100	98	85	76	121	100	95
	0.000	N/A	N/A	N/A	N/A	N/A	N/A	N/A
RT	197.380	100	99	93	87	107	104	101
	101.761	100	88	77	83	96	89	82
	43.270	100	100	82	81	115	104	92
	0.000	N/A	N/A	N/A	N/A	N/A	N/A	N/A

22. Matrix Effects: Lipemic and Hemolyzed Samples[XE "Matrix Effects"][XE "Matrix Effects"]

The assay was tested for matrix effects by spiking bone AP into lipemic and hemolyzed sera and calculating recovery on the Theranos system. Recovery of bone AP in lipemic and hemolyzed samples look reasonable during testing and these sample types are not expected to cause problems for the Theranos bone AP assay.

Spiked Lipemic Serum (ProMedDX), Theranos System

[Spiked] BAP U/L	Calculated [Activity] U/L	Activity % CV	% Recovery Relative to Nominal
81.16	83.58	5.5	103
63.88	57.84	10.6	91
38.62	28.35	5.8	73
21.24	18.21	12.0	86
17.93	16.93	15.8	94
10.73	10.46	5.5	97

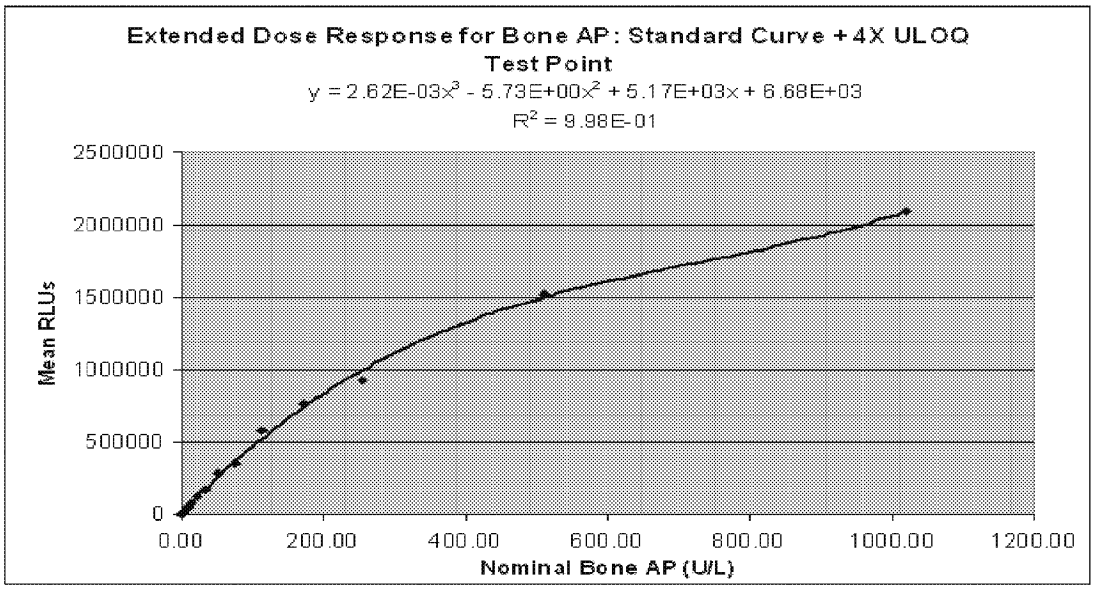
Spiked Hemolyzed Serum (ProMedDX), Theranos System

[Spiked] BAP U/L	Calculated [Activity] U/L	Activity % CV	% Recovery Relative to Nominal
81.16	79.72	13	98
63.88	61.66	7	97
38.62	43.16	3.0	112
21.24	20.54	9.1	97
17.93	17.51	7.6	98
10.73	10.85	13.3	101

23. Extended Range

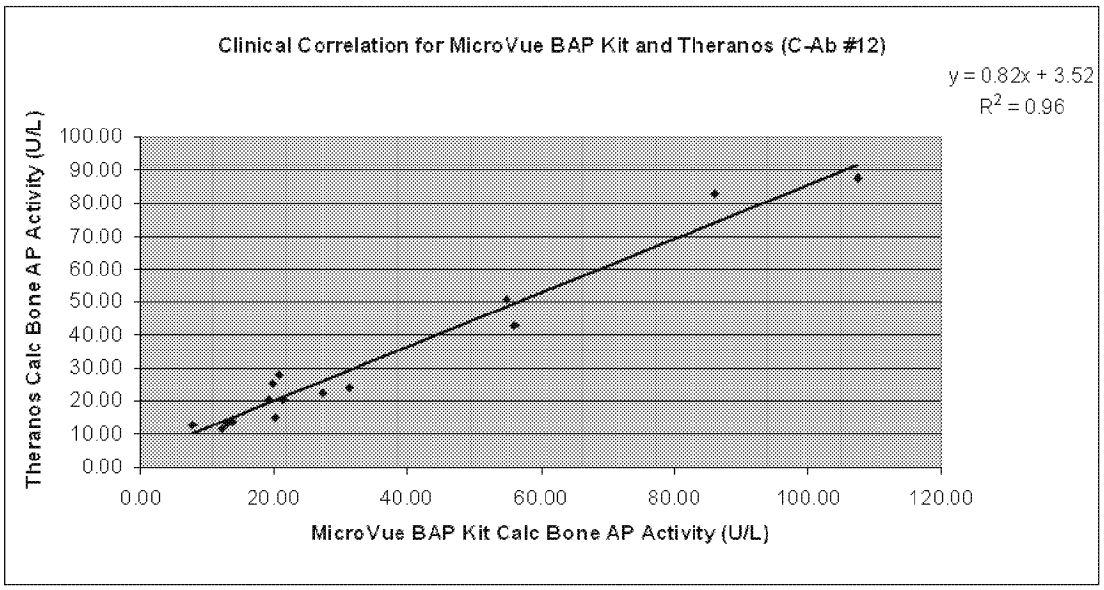
To determine the extended range of the bone AP linearity and dose response were tested for the standard curve with a point at 4X the current desired ULOQ. The tested 1022.4 U/L point only gave 78% recovery suggesting that the assay is probably not able to see out that far using the current calibration set. However, the point did still line up with the rest of the response curve showing some consistency even out past the calibrated assay range.

Spiked [BAP] U/L	Activity CV %	Mean Calc Activity (U/L)	% Recovery Relative to Nominal
1022.4	12.2	794.83	78



24. Assay Accuracy: Clinical Sample Assay Method Correlation [XE "Assay accuracy Clinical Sample Assay correlation"]

Clinical osteoporosis, renal insufficiency, and alkaline phosphatase samples obtained from ProMedDX were run in the Theranos system (calibrated using bone AP from CalZyme with activity assigned based on testing in the MicroVue BAP kit) and the MicroVue BAP ELISA by Quidel. For a total of 20 samples across the range of the assay, correlation was Theranos y = 0.82x + 3.52, R2 = 0.96 (see table below). For the set of 20 samples, the average Theranos system concentration CV was 7.5%. Theranos system conditions were the current best stated in this report, including testing on the Generic_10 protocol. Correlation was fairly good between Theranos and the MicroVue kit and the samples covered the relevant clinical range.



Sample #	MicroVue Kit Calc Bone AP Activity (U/L)	Theranos Calc Bone AP Activity (U/L)	%(Theranos/MicroVue)
22	50.42	35.26	70
23	57.11	47.18	83
24	37.61	38.53	102
25	55.84	41.84	75
32	19.76	25.32	128
33	7.88	12.70	161
38	20.77	27.95	135
41	13.72	13.61	99
42	27.32	22.60	83
43	21.30	20.30	95
44	19.23	20.63	107
45	13.72	14.24	104
46	12.12	11.51	95
47	12.99	13.34	103
49	20.14	15.37	76
50	31.25	24.08	77
51	54.85	51.16	93
52	86.05	82.81	96
53	107.56	87.52	81
54	56.17	43.09	77

25. Conclusions[XE "Conclusions"]

The current bone AP assay still has a few experiments left to complete but appears to perform well.