Development of Multiplexed Immunoassay Panels for Biochemical Markers of Bone Development

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Meso Scale Discovery has developed electrochemiluminescence-based multiplexed immunoassay panels to assay for serum/plasma levels of eight human markers of bone development, using the MSD MULTI-ARRAY™ platform, MSD MULTI-SPOT® 96-well plates and SECTOR™ instrumentation. The assays are distributed across 4 panels. Panel I measures levels of human bone alkaline phosphatase, osteoprotegerin, and sclerostin, using 25 µL per well of serum/plasma. The MSD assay provides a direct indication of protein levels in samples, in contrast to traditional activity based enzymatic assays. Panel II measures levels of osteocalcin, osteonectin, and osteopontin using 25 µL of a 20-fold diluted serum/plasma sample per well. The collagen processing by-product, procollagen Type I C-terminal peptide (PICP), a marker of bone formation, is measured in a singleplex assay using 25 µL per well of a 250-fold diluted serum/plasma sample. The growth factor TGFβ1, the most abundant growth factor in human bone, is measured in singleplex using acid-treated samples diluted 8-fold prior to addition to the assay plate. Thus, all eight biomarkers can be measured using less than 70 µL of sample for duplicate determinations. The assay formats are simple. Equal volumes of sample and assay diluent are added to assay plates and incubated for two-hours with agitation. The plates are washed, detection reagents are added, and the plates are incubated for an additional 1 hour incubation. After a final wash, MSD Read Buffer is added, and the plates are read on an MSD SECTOR Imager 6000 instrument (throughput of 1 plate per minute).
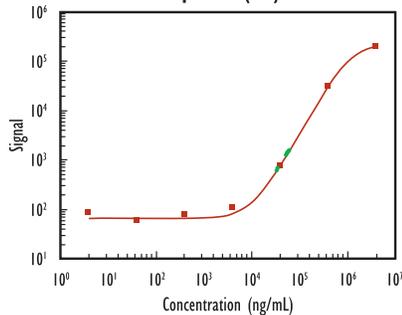
Assay ranges observed are 8-1,000 ng/mL for alkaline phosphatase, 6-20,000 pg/mL for osteoprotegerin, 1-10,000 pg/mL for sclerostin, 1-400 ng/mL for osteocalcin, 14-4,000 ng/mL for osteonectin, 0.5-400 ng/mL for osteopontin, 1->4,000 ng/mL for PICP, and 80-80,000 pg/mL for TGFβ1. The assays are sensitive enough to measure these biomarkers in normal samples, and the wide dynamic ranges should accurately measure elevated and decreased levels of the biomarkers in disease states. Spike recovery and dilution linearity were in the range of 75-115% for all assays tested, at most dilutions. In conclusion, four assay panels were developed and validated for measurement of eight bone-related biomarkers.

Meso Scale Discovery Applications

Assay Protocols

- Block MSD MULTI-SPOT plate for 1 hour, wash
- Add 25 µL of assay diluent solution to each well
- Add 25 µL of calibrator or sample (undiluted for Panel I; 20-fold diluted for Panel II; 250-fold for PICP assay; and 20-fold for TGFβ1 assay) to each well
- Incubate with shaking for 120 minutes, wash
- Add 25 µL of labeled antibody solution to each well
- Incubate with shaking for 60 minutes, wash
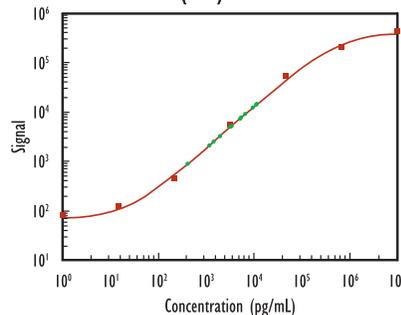- Add MSD Read Buffer
- Read plate on MSD Reader

Assay Performances: Panel I
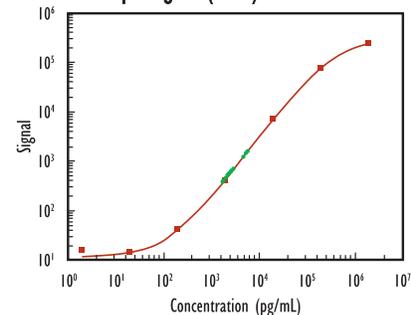
Bone Alkaline Phosphatase (ALP) Calibration Curve

Detection Limit:	8 ng/mL
Dynamic Range:	8-1,000 ng/mL

Sclerostin (SOST) Calibration Curve

Detection Limit:	1 pg/mL
Dynamic Range:	1-10,000 pg/mL

Osteoprotergerin (OPGN) Calibration Curve

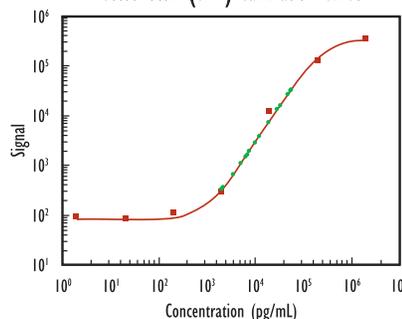| Detection Limit:	6 pg/mL
Dynamic Range:	6-20,000 pg/mL

Calibration curves (red) and sample data (N=5 each of human serum and matched heparin and EDTA plasma samples, values shown in green).

Detection limits are derived from a 4-parameter logistical fit of the calibrator values.

Assay Performances: Panel II

Osteonectin (ONN) Calibration Curve

Detection Limit:	715 pg/mL
Dynamic Range:	715-200,000 pg/mL

Osteocalcin (OCL) Calibration Curve

Detection Limit:	50 pg/mL
Dynamic Range:	50-20,000 pg/mL

Osteopontin (OPN) Calibration Curve

Detection Limit:	25 pg/mL
Dynamic Range:	25-20,000 pg/mL

Calibration curves (red) and sample data (20-fold dilution of N=5 each of human serum and matched heparin and EDTA plasma samples, values shown in green).

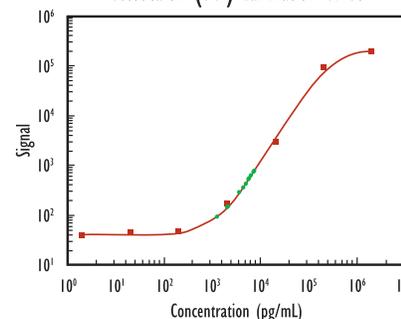Detection limits are derived from a 4-parameter logistical fit of the calibrator values and are not corrected for sample dilution.

Assay Performances

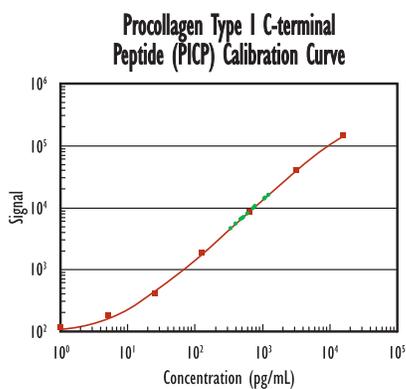## PICP Assay

Procollagen Type I C-terminal Peptide (PICP) Calibration Curve

Detection Limit:	4 pg/mL
Dynamic Range:	4>16,000 pg/mL

Calibration curve (red) and sample data (250-fold dilution of N=5 each of human serum & matched heparin and EDTA plasma samples, values shown in green).

TGFβ1 Assay

Transforming Growth Factor β 1 (TGFβ1) Calibration Curve

Detection Limit:	17 pg/mL
Dynamic Range:	20-10,000 pg/mL

Calibration curve (red) and sample data (8-fold dilution of N=7 of acid treated human serum, values shown in green).

Detection limits are derived from a 4-parameter logistical fit of the calibrator values and are not corrected for sample dilution.

Performance Summary
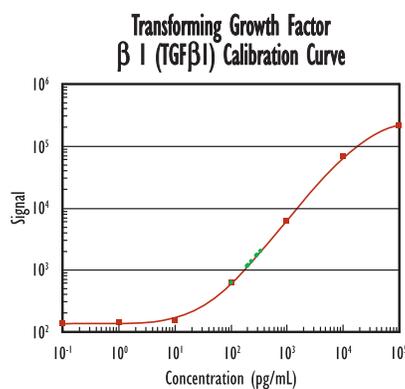
Assay	Assay Range*	Detection Limit	Sample Dilution Factor	Detection Limit (in sample)	Sample	Average Sample Concentration (n=4 or 5)
ALP	Serum	8 ng/mL	-	8 ng/mL	Serum	53 ng/mL
	Heparin Plasma				Heparin Plasma	54 ng/mL
	EDTA Plasma				EDTA Plasma	51 ng/mL
OPGN	Serum	6 pg/mL	-	6 pg/mL	Serum	275 pg/mL
	Heparin Plasma				Heparin Plasma	282 pg/mL
	EDTA Plasma				EDTA Plasma	299 pg/mL
SOST	Serum	1 pg/mL	-	1 pg/mL	Serum	85 pg/mL
	Heparin Plasma				Heparin Plasma	186 pg/mL
	EDTA Plasma				EDTA Plasma	162 pg/mL
OCL	Serum	50 pg/mL	20-fold	1 ng/mL	Serum	10 ng/mL
	Heparin Plasma				Heparin Plasma	11 ng/mL
	EDTA Plasma				EDTA Plasma	8 ng/mL
ONN	Serum	715 pg/mL	20-fold	14 ng/mL	Serum	360 ng/mL
	Heparin Plasma				Heparin Plasma	403 ng/mL
	EDTA Plasma				EDTA Plasma	410 ng/mL
OPN	Serum	25 pg/mL	20-fold	0.5 ng/mL	Serum	16 ng/mL
	Heparin Plasma				Heparin Plasma	18 ng/mL
	EDTA Plasma				EDTA Plasma	20 ng/mL
PICP	Serum	4 pg/mL	250-fold	1 ng/mL	Serum	371 ng/mL
	Heparin Plasma				Heparin Plasma	338 ng/mL
	EDTA Plasma				EDTA Plasma	337 ng/mL
TGFβ1	Serum	17 pg/mL	8-fold	0.13 ng/mL	Serum	2 ng/mL
	Heparin Plasma				Heparin Plasma	20 ng/mL
	EDTA Plasma				EDTA Plasma	30 ng/mL

*Assay range is from detection limit to upper end of linear range.

Dilution Linearity

Assay	Average Recovery at 3/4 Dilution Factor	Average Recovery at 1/2 Dilution Factor	Average Recovery at 1/4 Dilution Factor
ALP	109% $\pm$ 5%	113% $\pm$ 8%	147% $\pm$ 22%
OPGN	90% $\pm$ 5%	86% $\pm$ 16%	101% $\pm$ 25%
SOST	95% $\pm$ 8%	101% $\pm$ 7%	109% $\pm$ 14%
OCL	91% $\pm$ 2%	85% $\pm$ 5%	88% $\pm$ 6%
ONN	91% $\pm$ 5%	83% $\pm$ 11%	77% $\pm$ 9%
OPN	88% $\pm$ 4%	85% $\pm$ 6%	76% $\pm$ 9%
PICP	97% $\pm$ 15%	104% $\pm$ 15%	109% $\pm$ 17%
TGFβ1*	99% $\pm$ 14%	107% $\pm$ 14%	133% $\pm$ 14%

*-serum only

Serum, heparin plasma, and EDTA plasma samples from 5-6 individuals each were diluted with the appropriate calibrator diluents for each panel. Recoveries were measured at three dilution levels — the average recovery at each dilution level is tabulated.

Spike Recovery

Assay	Serum	Heparin Plasma	EDTA Plasma
ALP	106% $\pm$ 29%	98% $\pm$ 23%	82% $\pm$ 23%
OPGN	93% $\pm$ 16%	90% $\pm$ 13%	92% $\pm$ 13%
SOST	90% $\pm$ 11%	86% $\pm$ 9%	86% $\pm$ 5%
OCL	105% $\pm$ 9%	109% $\pm$ 3%	109% $\pm$ 11%
ONN	113% $\pm$ 9%	114% $\pm$ 6%	112% $\pm$ 10%
OPN	93% $\pm$ 8%	98% $\pm$ 4%	90% $\pm$ 10%
PICP	ND	ND	ND
TGFβ1*	95% $\pm$ 9%	ND	ND

ND - not determined   *-serum only, n=16

Serum and plasma samples from 5-6 different individuals were spiked with known amounts of ALP, OPGN, SOST, OCL, ONN, OPN, or TGFβ1 at defined levels (2-20 fold above native levels) and quantitated on the appropriate bone marker assay panels. The average recovery in each matrix is tabulated.

Conclusions

- Immunoassays for the bone development biomarkers bone alkaline phosphatase, osteoprotegerin, sclerostin, osteocalcin, osteonectin, osteopontin, PICP, and TGFβ1 have been demonstrated on MSD's MULTI-ARRAY platform.

- The assays are formulated on 4 panels based on sample dilution or sample preparation (TGFβ1 – acid activation of samples) differences.

- Assays are rapid (3 hours), easy to run, use small sample volumes, are sensitive, and have wide dynamic ranges (2 to >5 log).

- Assay sensitivity and wide dynamic range (2-5 logs) allow for measurement of normal and elevated levels without requiring measurement of serial dilution.