Development of Multiplex Immunoassay Panels for Simultaneous Quantification of Bone Metabolism Markers using Luminex® xMAP® Technology

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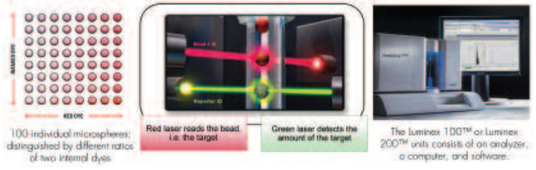
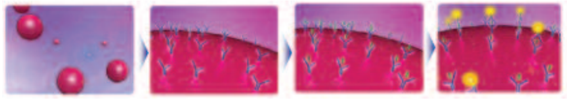
Abstract

Biochemical markers of bone metabolism play an important role in the assessment of bone diseases such as osteoporosis, arthritis, chronic inflammatory disorders, and bone metastasis with cancers. Millipore has developed various multiplexed immunoassay panels for the simultaneous measurement of multiple bone metabolism markers in mouse, rat, and human, using the Luminex® xMAP® platform. These bead-based sandwich assays are rapid, sensitive, and reproducible and require ≤ 25 µL of serum/plasma sample or tissue culture supernatants per well in 96-well plates. The mouse bone panels allow simultaneous quantitation of the following biomarkers in any combinations in the same sample well: Mouse Panel 1 - OPG, Osteocalcin, ACTH, Insulin, Leptin, TNFα, IL-6, IL-1β and Mouse Panel 2 - RANKL, Osteocalcin, ACTH, Insulin, Leptin, TNFα, IL-6, IL-β. The dynamic ranges for the mouse analytes are 10-40,000 pg/mL (OPG, Osteocalcin, RANKL, Leptin), 24-100,000 pg/mL (Insulin), and 2-10,000 pg/mL (ACTH, TNFα, IL-6, IL-β). The rat bone panels allow simultaneous measurement of the following biomarkers: Rat Panel 1 - OPG, ACTH, Insulin, Leptin, Rat Panel 2 - RANKL, ACTH, Insulin, Leptin, and Rat Panel 3 – Osteocalcin, PTH, Osteopontin. The dynamic ranges for the rat analytes are 10-40,000 pg/mL (OPG, PTH, Leptin), 5-20,000 pg/mL (Osteocalcin), 4-15,000 pg/mL (RANKL), 24-100,000 pg/mL (insulin), and 2-10,000 pg/mL (ACTH). The human bone metabolism panels allow for simultaneous measurement of the following biomarkers in any combinations: Human Panel 1A serum samples) - OPG, Osteocalcin, Osteopontin, PTH, Leptin, ACTH, Insulin and Human Panel 1B (tissue culture samples) - OPG, Osteocalcin, Osteopontin, PTH, Leptin, Adiponectin, Insulin, ACTH, TNFα, IL-6, IL-βu. We have also developed a sensitive immunoassay for measuring the serum levels of human RANKL. The dynamic range for this Human RANKL Single-plex assay is 5-20,000 pg/mL. The above bone metabolism panels exhibited acceptable analytical performance characteristics in terms of sensitivity, intra- and inter-assay precision, linearity of dilution, spike recovery, and antibody pair specificity. In conclusion, various multiplexed assay panels were developed and validated for the measurement of multiple bone metabolism markers in mouse, rat, and human samples. The availability of these multiplexed Bone Metabolism Panels and the RANKL, OPG, and Osteocalcin Single-plex assays may provide a powerful tool in studying biological functions of these biomarkers as well as the pathological roles of these molecules.

Luminex® xMAP® Technolgy



Bone Panel Assay Method



Beads: Capture antibodies were covalently coupled to the carboxylate-modifed microsphere beads using EDC and Sulfo-NHS according to the manufacturer's instructions.

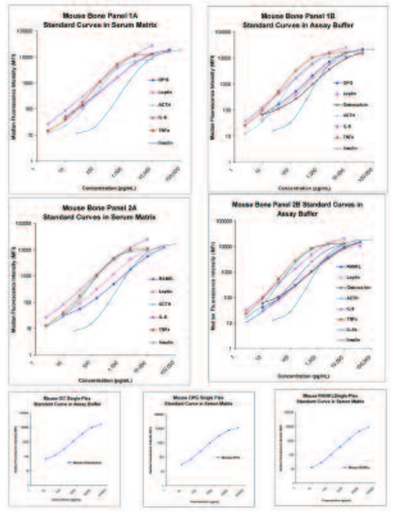
Assay Procedures: The multiplex assay was performed in a 96-well Millipore MultiScreen filter plate. The protocol is as follows: wet the plate with 200 µl assay buffer → 25 µl assay buffer. 25 µl standards/samples. 25 µl matrix/buffer, 25 µl beads → incubate at 4°C, overnight → wash plate 3 times → add 50 µl biotinylated secondary Abs, RT, 1 hr → add 50 µl Streptavidin-Phycoerythrin (SAPE), RT, 30 min → wash plate 3 times → read on Luminex instruments

Mouse Bone Metabolism Panels

7 Mouse Bone Panels: 4 Multiplex and 3 Single-plex

Mouse Panel 1A†	Mouse Panel 1B*	Mouse Panel 2A†	Mouse Panel 2B*	Mouse Single-plex
OPG Insulin Leptin ACTH IL-6 TNFα IL-1β‡	OPG Insulin Leptin ACTH IL-6 TNFα OC IL-1β‡	RANKL Insulin Leptin ACTH IL-6 TNFα IL-1β‡	RANKL Insulin Leptin ACTH IL-6 TNFα OC IL-1β‡	Mouse OC* OPG† RANKL†

†serum/plasma *tissue culture samples or >1:20 diluted serum/plasma ‡special release



Mouse Bone Panels: Assay Characteristics

ASSAY CONDITIONS: Overnight assay
25 µl or less sample volume

STANDARD CURVE RANGE:
10 – 40,000 pg/mL – Leptin, OPG, Osteocalcin, RANKL
24 – 100,000 pg/mL – Insulin
2 – 10,000 pg/mL – ACTH, IL-6, TNFα

SENSITIVITY
(Minimum Detectable Concentration, MinDC, pg/mL):

	1A	1B	2A	2B
RANKL	—	—	3.3	2.7
OPG	2.3	2.4	—	—
Insulin	18.6	17.4	16.2	14.4
Leptin	3.0	3.2	4.5	4.4
ACTH	1.8	1.4	1.7	1.3
IL-6	0.6	0.6	0.6	0.6
TNFα	1.0	0.8	0.8	0.6
Osteocalcin	—	8.5	—	4.7

ACCURACY (%):

	1A	1B	2A	2B
RANKL	—	—	119	78
OPG	95	90	—	—
Insulin	92	101	92	91
Leptin	87	93	93	93
ACTH	117	92	115	85
IL-6	86	87	89	90
TNFα	122	92	133	93
Osteocalcin	—	99	—	97

LINEARITY OF DILUTION:

1A	108-137%
1B	87-123%
2A	86-129%
2B	98-125%

PRECISION:
Intra-assay <4%
Inter-assay <11%

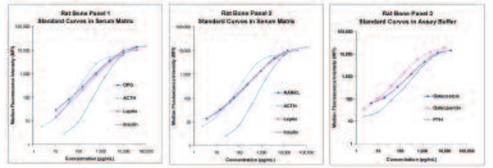
CROSS REACTIVITY: The antibody pairs in each panel do not show cross reactivity to the other analytes within the panel.

Rat Bone Metabolism Panels

6 Rat Bone Panels: 3 Multiplex and 3 Single-plex

Rat Panel 1†	Rat Panel 2†	Rat Panel 3*‡	Rat Single-plex
OPG Insulin Leptin ACTH	RANKL Insulin Leptin ACTH	OC OPN PTH	Rat OC* OPG† RANKL†

†serum/plasma or tissue culture *tissue culture or >1:50 diluted serum/plasma ‡special release



Rat Bone Panels: Assay Characteristics

ASSAY CONDITIONS: Overnight assay
25 µl or less sample volume

STANDARD CURVE RANGE:

ACTH	2.4 – 10,000 pg/mL
RANKL	3.7 – 15,000 pg/mL
OPG, Leptin	9.8 – 40,000 pg/mL
Insulin	24 – 100,000 pg/mL

SENSITIVITY (Minimum Detectable Concentration, MinDC, pg/mL):

	Panel 1	Panel 2
OPG	2.3	—
Insulin	18.6	16.2
Leptin	3.0	4.5
ACTH	1.8	1.7
RANKL	—	1.1

ACCURACY (%):

	Panel 1	Panel 2
OPG	78.6	—
Insulin	75.8	76.1
Leptin	78.0	87.7
ACTH	99.2	101.8
RANKL	—	118.4

LINEARITY OF DILUTION:

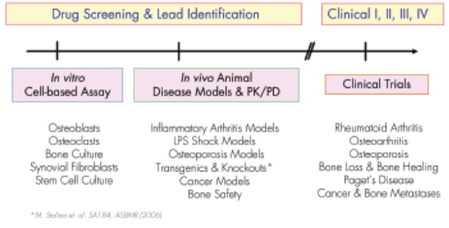
Panel 1	82-117%
Panel 2	94-120%

PRECISION:
Intra-assay <4%
Inter-assay <9%

CROSS REACTIVITY: The antibody pairs in each panel do not show cross reactivity to the other analytes within the panel.

Bone Panel Applications

Bone Biomarkers in Drug Development



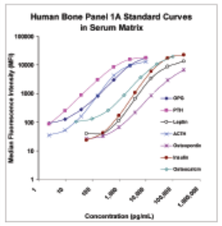
*M. Skaftos et. al. SA184, ASBMR(2006)

Human Bone Metabolism Panels

3 Human Bone Panels: 2 Multiplex and 1 Single-plex

Human Panel 1A†	Human Panel 1B*	Human Single-plex
OPG OC OPN PTH Leptin ACTH Insulin	OPG OC OPN PTH IL-1β TNFα IL-6 Leptin ACTH Adiponectin Insulin	RANKL ‡

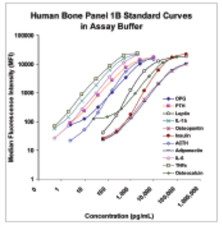
†1:4 diluted serum/plasma *tissue culture samples ‡1:2 diluted serum/plasma



Human Bone Metabolismm Panel 1A (in Serum Matrix)

Analyte	Sensitivity (pg/ml)	Accuracy (% Recorery)	Linearity (% Recovery)	Intra-Assay (% CV)	Inter-Assay (% CV)
OPG	1.42	96.4	105.0	6.5	6.3
OC	39.3	98.4	85.6	5.6	10.7
Leptin	119.7	136.3	138.0	4.8	6.2
OPN	67.8	79.7	126.5	5.4	5.8
PTH	0.30	98.1	117.5	7.0	9.6
ACTH	2.1	89.2	120.3	4.9	5.6
Insulin	46.3	95.2	104.1	3.5	6.3

Sensitivity: Minimum Detectable Concentration (MinDC)
Accuracy: Percentage of measured analytes in 4 independent samples spiked with 3 know concentration of analytes
Dilution Linerarity: Average percent recovery of 4 independent samples diluted 1:2, 1:4, and 1:8 in the kit provided matrix
Precision: Intra-assay precision is generated from the mean of the %CV from 16 reportable results across 2 different concentrations of analytes in a single assay. Interassay precision is generated fromt he mean of the %CV from 2 reportable results across 2 different concentrations of analytes across 6 different assays.
Cross Reactivity: The antibody pair in each panel do not show cross reactivity to the other analytes within the panel

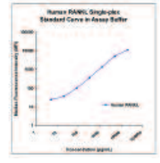


Human Bone Panel 1B (in Assay Buffer)

Analyte	Sensitivity (pg/ml)	Accuracy (% Recorery)	Linearity (% Recovery)	Intra-Assay (% CV)	Inter-Assay (% CV)
IL-1β	0.13	93.3	109.4	3.5	7.3
IL-6	0.09	94.5	94.5	3.9	6.1
OPG	0.90	102.5	104.0	3.2	4.5
OC	39.8	101.2	91.4	3.7	6.7
Leptin	18.8	94.0	118.4	`3.7	4.7
OPN	32.8	103.7	100.9	4.0	5.6
PTH	0.32	98.1	104.3	5.2	9.8
TNFa	0.06	101.2	101.7	4.8	5.9
ACTH	1.3	96.6	96.2	3.4	7.8
Adiponectin	22.2	96.9	97.4	3.3	10.2
Insulin	38.5	100.0	96.8	2.7	5.5

Human RANKL Single-plex (in Serum Matrix)

Analyte	Sensitivity (pg/ml)	Accuracy (% Recorery)	Linearity (% Recovery)	Intra-Assay (% CV)	Inter-Assay (% CV)
RANKL	4.8	93.2	121.7	<5.0	<6.0



Summary

- We have developed mouse, rat, and human specific multiplex immunoassay panels for simultaneous measurement of bone metabolism markers. Analytic parameters evaluated show the robustness of the assay.
- The assay is rapid, sensitive, and reproducible and only 25 µL of sample is required.
- The availability of Millipore multiplex bone metabolism panels provides a useful tool in studying biological functions of these bone biomarkers and the pathological roles of these molecules.