Cat. # MK126

For Research Use

TaKaRa

Rat Gla-Osteocalcin High Sensitive EIA Kit

Product Manual

v201903Da

Table of Contents

I. Description

Osteocalcin, which contains two or three γ-carboxyglutamate (Gla) residues and has a molecular weight of approximately 5,900 Daltons, is known as a vitamin K-dependent calcium-binding non-collagen protein. Specifically produced by only osteoblasts, osteocalcin has been used as one of the osteoblast markers. The rat osteocalcin consists of a total of 50 amino acids. Human, bovine, rabbit, and other species have a osteocalcin with 49 amino acids. The three glutamate residues at positions 17, 21, and 24 of the amino acid chain are carboxylated, forming a calcium pocket that allows osteocalcin to bind to bone matrix.

Osteoblasts generally produce osteocalcin with all three of its glutamate residues carboxylated (Gla-OC), affording the protein its ability to bind with bone matrix. During bone metabolism, osteocalcin is released from bone matrix through the actions of various enzymes, including one produced by osteoclasts. Most of the three glutamate residues are decarboxylated on osteocalcin (Glu-OC) when it is released into blood from bone. Therefore, osteocalcin is present in blood in both Gla and Glu forms and is made up of a wide range of molecular species, from full-length to fragmented molecules.

```
                    10        20        30        40        50
Human     1  YLYQWLGAPV PYPDPLEPRR EVCELNPDCD ELADHIGFQE AYRRFYGP-V
Bovine    1  YLDHWLGAPA PYPDPLEPKR EVCELNPDCD ELADHIGFQE AYRRFYGP-V
Rat       1  YLNNGLGAPA PYPDPLEPHR EVCELNPNCD ELADHIGFQD AYKRIYGTTV
Mouse     1  YL----GASV PSPDPLEPTR EQCELNPACD ELSDQYGLKT AYKRIYGITI
Chicken   1  YAQDSGVAGA P-PNPLEAQR EVCELSPDCD ELADQIGFQE AYRRFYGP-V
Monkey    1  YLYQWLGAPA PYPDPLEPKR EVCELNPDCD ELADHIGFQE AYRRFYGP-V
Pig       1  YLDHGLGAPA PYPDPLEPRR EVCELNPDCD ELADHIGFQE AYRRFYGI-A
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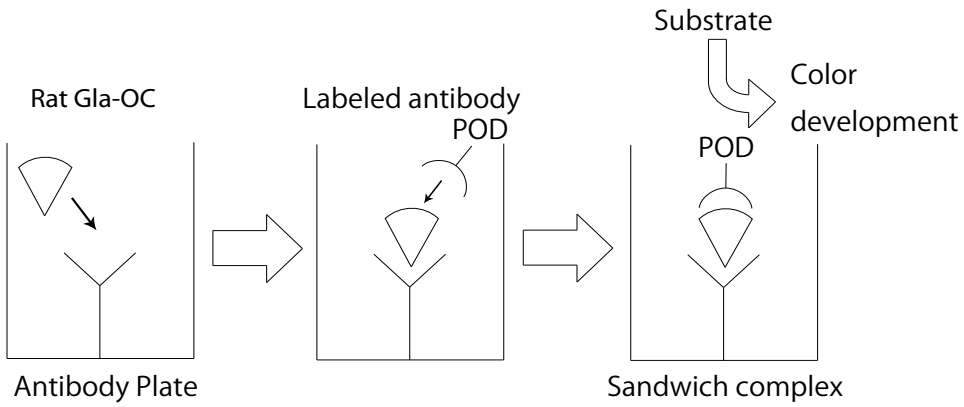
Figure 1. The Amino Acid Sequence (Primary Structure) of Osteocalcin in Animal Species

Rat Gla-Osteocalcin High Sensitive EIA Kit is a sandwich-type EIA kit, which uses a rat osteocalcin C-terminus-specific antibody as capture-antibody on a solid-phase plate. This antibody has a minimal cross reactivity with bovine, human and rabbit osteocalcin. An enzyme-labeled antibody (GlaOC4-30) specific to Gla-OC is used as the detection antibody, allowing this kit to detect Gla-osteocalcin with a very high sensitivity. As such, this EIA kit is sensitive enough to detect even minute levels of rat osteocalcin produced in supernatants of cells cultured in fetal calf serum-supplemented medium.

II. Principle



III. Components

(1) Antibody Coated Microtiter plate — 1 plate
Anti-Rat OC monoclonal antibody
(96 wells：8 wells x 12 strips)

(2) Antibody-POD Conjugate (lyophilized) — for 11 ml
Peroxidase-labeled anti-Gla-OC monoclonal antibody

(3) Standard (lyophilized) — for 1 ml
Gla Osteocalcin Full-length Peptide 16 ng

(4) Sample Diluent — 11 ml x 2
BlockAce-containing PBS (with preservative)

(5) Substrate Solution (TMBZ) — 12 ml
3,3',5,5'-Tetramethylbenzidine solution

IV. Materials Required but not Provided

- Wash and Stop solution for ELISA without Sulfuric Acid (Cat. #MK021)
  Contains wash solution (10X PBS, 50 ml x 5 tubes; Tween 20, 3 ml) and reaction stop solution (60 ml).
  ＊ This product is a stop solution for peroxidase reactions without 1N sulfuric acid.
  ＊ 1N sulfuric acid can be used as a stop solution. Handle 1N sulfuric acid with caution.
- Pipette, micropipette, and tips
- Microplate reader (capable of measuring absorbance of up to 3.5 when set to 450 nm)

V. Storage

4℃

VI. Intended Use

In vitro enzyme immunoassay (EIA) for quantitative determination of Gla-osteocalcin (Rat Gla-OC) in rat-derived biological samples.

VII. Protocol

1. Sample

- Suitable samples include rat serum, ascites, cell culture supernatant, and cell extracts, etc.
- Samples may be stored up to 12 hours at 4℃. If the assay will be performed longer than 12 hours after sample preparation, then store samples frozen at -20℃.
- Use Sample Diluent (4) for dilution if necessary.
- The recommended dilution for rat serum samples is 50 - 200-fold.

2. Preparation of Solutions

- Antibody Coated Microtiter Plate
  Allow the (1) Anti-Rat OC Specific Monoclonal Antibody plate to reach room temperature unopened in its package before use.
- POD-labeled Antibody Solution
  Reconstitute (2) Antibody - POD Conjugate with 11 ml of distilled water.
  Once reconstituted, it is stable for up to 1 week at 4℃. For longer storage, freeze at -20℃, at which it is stable for up to 1 month. Once thawed, it may not be returned to frozen storage.
- Rat Gla-OC Standard Solution
  Add 1 ml of distilled water to the (3) Rat serum Gla-osteocalcin to reconstitute the Standard (16.0 ng/ml). Dilute it with (4) Sample Diluent before use to prepare fresh serial dilutions of Standard Solution at concentrations of 16.0, 8.0, 4.0, 2.0, 1.0, 0.5, and 0.25 ng/ml. Use Sample Diluent as the 0-concentration standard.
  The Rat Gla-OC Standard Solution (16.0 ng/ml) is stable for up to 1 week after preparation when stored at 4℃, or for up to 1 month at -20℃.
- Substrate Solution
  Return (5) Substrate Solution (TMBZ) to room temperature before use. It is supplied ready to use. Check before use that the Substrate Solution has not developed a dark blue color. A reaction with metal ions will result in coloration; make sure it is not contaminated with any tap water.
  If the Substrate Solution will be used for several assays, divide it into aliquots of the required volume in advance.
- Stop solution
  Use the Stop solution included in Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021) directly.
  ＊ Because this is highly viscous, mix well using a plate mixer after its introduction.
- PBS with 0.1% Tween 20 for washing
  Dilute the 10X PBS included in Wash and Stop solution for ELISA without Sulfuric Acid (Cat. #MK021) 10 fold with distilled water, and then add Tween 20 to a final concentration of 0.1%.
  For 96 reactions performed with this kit, 300 ml of washing solution is required.

3. Procedure

Assay samples in duplicate.
Return each reagent in the kit and samples to room temperature and make sure solutions are mixed uniformly without creating bubbles before use.

1. Prepare reagents and samples (100 µl each) in a separate 96-well plate in advance so that they can be added to the antibody-plate quickly (within 5 min) using an 8-channel pipette or similar apparatus. In order to provide highly reliable results, it is recommended to place serial dilutions of the Standard Solution in the 1st and 12th rows. Perform this reaction at room temperature (20 - 30℃) for 1 hour; incubation at 37℃ may compromise antigenicity. [First reaction]
2. Discard reaction mixtures, followed by 3 washes with Washing Buffer. Then add 100 µl of the labeled antibody solution per well using an 8-channel pipette and allow to react for 1 hour at room temperature (20 - 30℃). [Second reaction]
3. Discard reaction mixtures, followed by 4 washes with Washing Buffer. Then add 100 µl of (5) Substrate Solution (TMBZ) per well using an 8-channel pipette and allow to react at room temperature (20 - 30℃) for 10 - 15 min. [Third reaction]
4. Add 100 µl of Stop Solution to each well to stop the reaction in the same order as for (5) Substrate Solution (TMBZ). Then mix well.
5. Use distilled water as a control to make zero adjustment and measure absorbance at 450 nm.

   The color is stable for up to 1 hour after reaction termination.
6. Plot a standard curve based on the results obtained from the Standard Solutions (with concentration as x-axis and absorbance as y-axis) and use it to determine the corresponding concentrations of Rat Gla-OC based on the sample's absorbance.

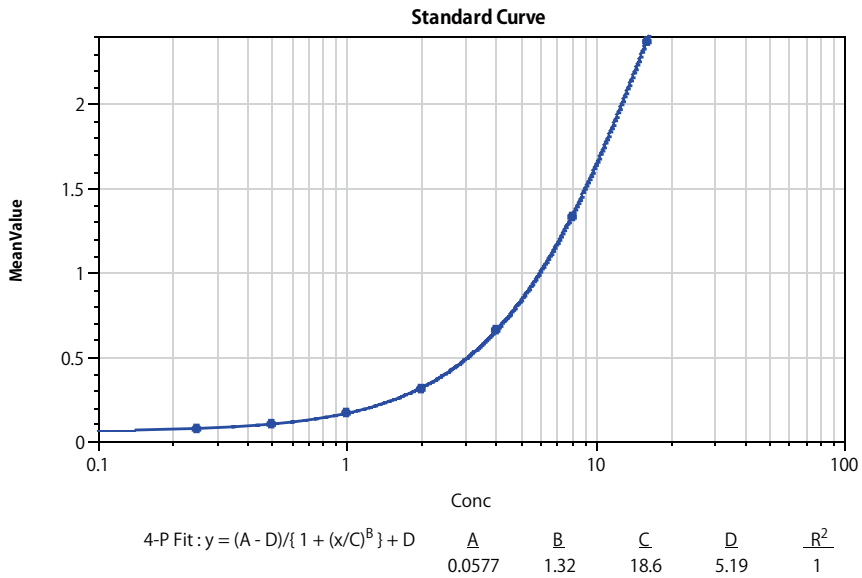
Note:

- Cover the plate with film or the like to prevent evaporation of solutions during reactions at room temperature or in an incubator.
- It is recommended that the Washing Buffer be completely discarded to get rid of the residual fluid.

VIII. Performance

1. Standard curve (Rat Gla-Osteocalcin High Sensitive EIA Kit)

The following shows a typical standard curve of this kit as an example.
The standard curve for calculation needs to be established in each assay.



Rat Gla-OC (ng/ml)	16.0	8.0	4.0	2.0	1.0	0.5	0.25	0.0
Absorbance A_{450}	2.372	1.332	0.659	0.313	0.169	0.105	0.079	0.052

(Color Development Time : 15 min)

2. Reproducibility

<Intra-assay precision test (n=16)>

A reproducibility test was performed with 16 replicates, using 3 different concentrations of rat serum.

Sample	Mean (ng/ml)	CV (%)
Control A	4.157	5.4
Control B	2.521	3.8
Control C	1.130	5.0

<Inter-assay precision test (n=3)>

The reproducibility test was performed with triplicates, by assaying 3 different concentrations of sample over 3 days.

Sample	Mean (ng/ml)	CV (%)
Control A	4.358	4.6
Control B	2.318	7.7
Control C	1.127	2.2

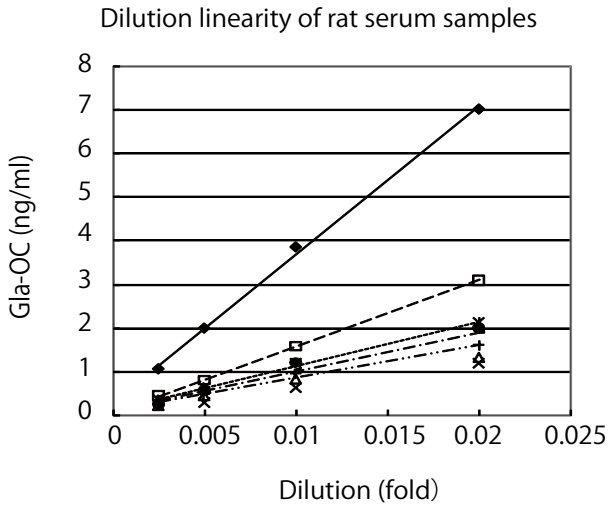
3. Recovery test

Equal volumes of samples in different concentrations were combined and assayed. The result of each mixture was compared with the theoretical value to detemine the recovery rate.

Sample A	Sample B	A+B (Theoretical Value)	A+B (Assay Result)	Recovery Rate (%)
4.145	1.805	2.975	2.945	98.99
4.145	1.523	2.834	2.529	89.24
4.145	1.211	2.678	2.575	96.15
4.145	0.752	2.449	2.180	89.03
4.145	0.729	2.437	2.141	87.85
4.145	0.630	2.388	2.251	94.28
4.145	0.394	2.270	2.022	89.09
1.805	1.523	1.664	1.803	108.35
1.805	1.211	1.508	1.482	98.28
1.805	0.752	1.267	1.339	105.68
1.805	0.630	1.218	1.227	100.78
1.805	0.394	1.100	1.096	99.68
1.523	1.211	1.367	1.606	117.48
1.523	0.752	1.138	1.356	119.21
1.523	0.729	1.126	1.223	108.61
1.523	0.630	1.077	1.110	103.11
1.523	0.394	0.959	1.122	117.06
0.752	1.211	0.982	1.000	101.88
0.752	0.630	0.691	0.717	103.76
0.752	0.394	0.573	0.650	113.44
0.729	1.211	0.970	0.927	95.57
0.729	0.752	0.741	0.731	98.72
0.729	0.630	0.680	0.670	98.60
0.729	0.394	0.562	0.531	94.57
0.394	1.211	0.803	0.793	98.82

n=26, Units =ng/ml

4. Linearity of rat serum



◆ [1]	y=339.2x + 0.314	r^2=0.9981
□ [2]	y=154.01x + 0.0549	r^2=0.9999
△ [3]	y=62.393x + 0.1366	r^2=0.9825
× [4]	y=60.643x + 0.0115	r^2=0.9972
∗ [5]	y=101.04x + 0.1398	r^2=0.9975
● [6]	y=99.475x + 0.1112	r^2=0.9848
+ [7]	y=76.01x + 0.1267	r^2=0.992
− [8]	y=88.525x + 0.1418	r^2=0.9994

5. Effects of freeze-thaw cycle and incubation on samples

The effect of freeze-thaw cycle on the concentration of rat Gla-OC was studied. Rat serum, ascites, and purified osteocalcin were subject to freeze-thaw cycles between 2 temperatures; 25℃ and -80℃. Assay samples were collected after each thaw. All samples were assayed at the same time after the final samples were collected. To study the effect of incuabation, samples were incubated for 1 or 2 hours at 37℃ before undergoing quantitative determination.

Freeze-Thaw	Purified Osteocalcin	Rat Serum [1]	Rat Serum [2]	Rat Ascites [1]
0 cycle	6.935	1.217	0.378	1.085
1 cycle	8.198	2.293	0.771	1.301
2 cycle	8.233	2.331	0.731	1.211
3 cycle	8.525	2.397	0.832	1.292
4 cycle	9.041	2.590	0.794	1.253
5 cycle	8.963	2.599	0.790	1.241

Incubation at 37℃	Std (8 ng/ml)	Rat Serum [1]	Rat Serum [2]	Rat Ascites [1]
1 hour	7.357	0.969	0.304	1.043
2 hours	7.150	0.934	0.306	1.217

Unit: ng/ml

Result:
Incubation at 37℃ resulted in a number of samples having a low concentration. This is likely due to enzyme degradation by protease contamination. In addition, it is necessary to keep the number of freeze-thaw cycles the same across all samples.

6. Cross reactivity with various animal serum samples

Species	Chicken		Duck		Dove		Turkey	
Dilution rate	A_{450}	ng/ml	A_{450}	ng/ml	A_{450}	ng/ml	A_{450}	ng/ml
x 5	0.077	0.084	0.065	ND	0.080	0.124	0.062	ND
x 25	0.069	ND	0.056	ND	0.065	ND	0.055	ND
x 125	0.057	ND	0.058	ND	0.058	ND	0.059	ND
x 625	0.060	ND	0.061	ND	0.063	ND	0.064	ND

Species	Goose		Horse		Goat		Bovine	
Dilution rate	A_{450}	ng/ml	A_{450}	ng/ml	A_{450}	ng/ml	A_{450}	ng/ml
x 5	0.073	ND	0.199	1.035	0.086	0.193	0.157	0.762
x 25	0.069	ND	0.112	0.431	0.074	0.035	0.090	0.234
x 125	0.062	ND	0.082	0.148	0.065	ND	0.066	ND
x 625	0.062	ND	0.065	ND	0.063	ND	0.056	ND

ND: not detectable

Sample	Human		Cynomolgus Monkey		ICR Mouse		Guinea Pig	
Dilution rate	A_{450}	ng/ml	A_{450}	ng/ml	A_{450}	ng/ml	A_{450}	ng/ml
x 5	0.072	ND	0.097	0.301	0.796	4.242	0.087	0.204
x 25	0.067	ND	0.077	0.084	0.258	1.388	0.074	0.035
x 125	0.056	ND	0.062	ND	0.086	0.193	0.062	ND
x 625	0.061	ND	0.058	ND	0.065	ND	0.053	ND

Sample	Rabbit		Human No. 11		Human No. 12		Human No. 13	
Dilution rate	A_{450}	ng/ml	A_{450}	ng/ml	A_{450}	ng/ml	A_{450}	ng/ml
x 5	0.299	1.621	0.156	0.755	0.084	0.171	0.080	0.124
x 25	0.189	0.972	0.095	0.283	0.069	ND	0.072	ND
x 125	0.116	0.463	0.065	ND	0.058	ND	0.064	ND
x 625	0.078	0.098	0.060	ND	0.061	ND	0.057	ND

Sample	Human No. 14		Human No. 15	
Dilution rate	A_{450}	ng/ml	A_{450}	ng/ml
x 5	0.495	2.671	0.080	0.124
x 25	0.127	0.548	0.067	ND
x 125	0.077	0.084	0.057	ND
x 625	0.063	ND	0.057	ND

<Rat Samples>

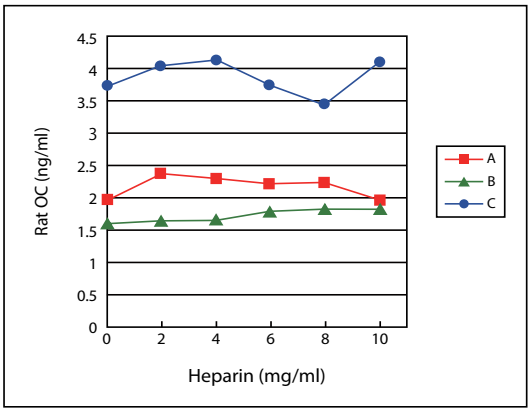
Rat Serum	Serum No. 13		Serum No. 16		Serum No. 17		Serum No. 18	
Dilution rate	A_{450}	ng/ml	A_{450}	ng/ml	A_{450}	ng/ml	A_{450}	ng/ml
x 50	1.226	7.029	0.525	3.133	0.217	1.346	0.198	1.215
x 100	0.662	3.868	0.258	1.605	0.147	0.846	0.122	0.646
x 200	0.324	2.007	0.142	0.810	0.102	0.461	0.087	0.296
x 400	0.177	1.072	0.100	0.447	0.082	0.233	0.070	ND

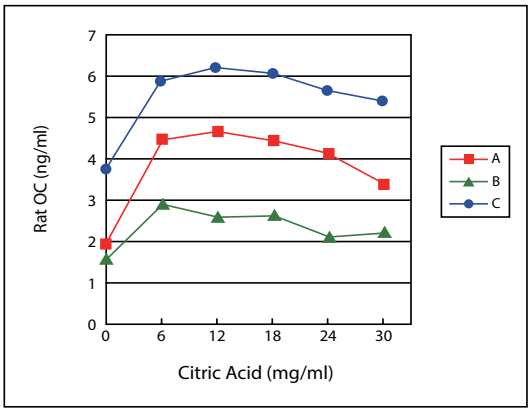
Rat Ascites	Ascites No. F2		Ascites No. F3		Ascites No. F4		Ascites No. F5	
Dilution rate	A_{450}	ng/ml	A_{450}	ng/ml	A_{450}	ng/ml	A_{450}	ng/ml
x 50	0.347	2.138	0.330	2.043	0.259	1.615	0.308	1.910
x 100	0.195	1.198	0.202	1.244	0.162	0.963	0.173	1.040
x 200	0.124	0.659	0.117	0.598	0.106	0.501	0.113	0.562
x 400	0.092	0.353	0.086	0.290	0.085	0.278	0.094	0.375

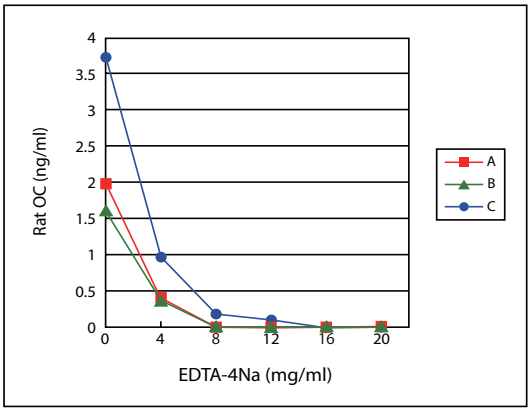
ND: not detectable

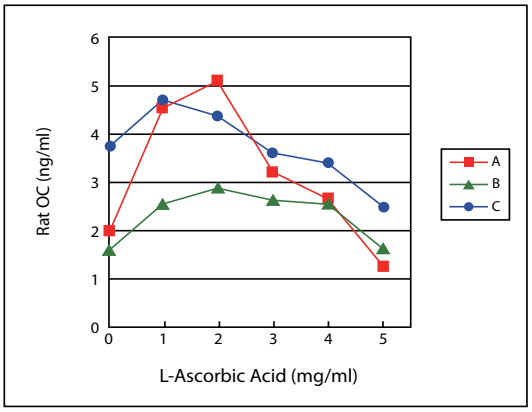
7. Effects of coexisting substances in samples

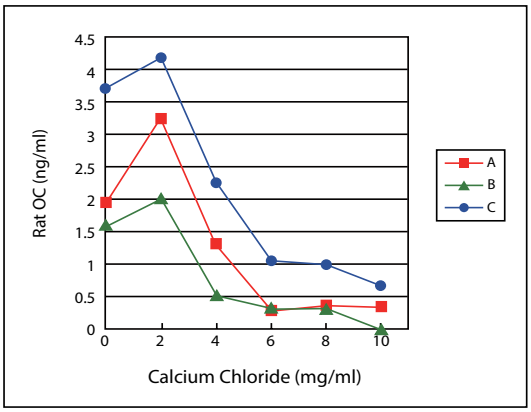
Three different concentrations (A, B and C) of osteocalcin standard solutions were used. Each standard solution was combined with a test material at a ratio of 9 to 1 to investigate the test material's effect on the reaction system. Test material concentrations shown in the graphs are final concentrations.











Result:
EDTA-4Na and calcium chloride show an interfering tendency; care should be paid to avoid introduction of these substances into samples.

8. Correlation with Rat Gla-OC Competitive EIA Kit

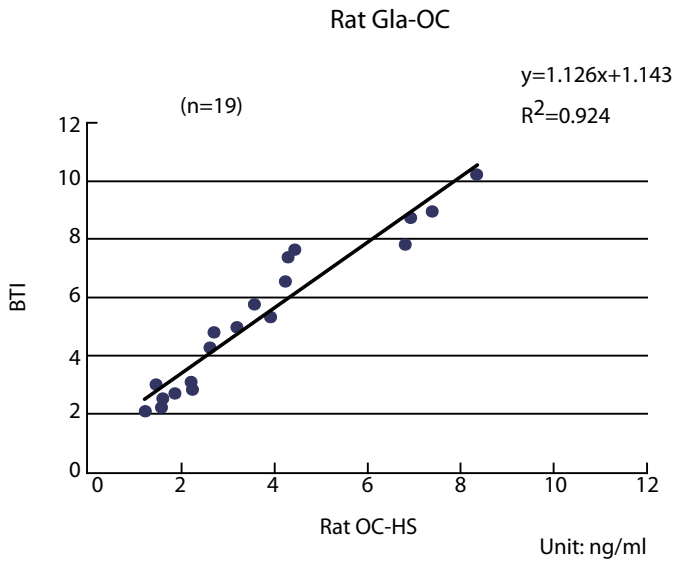
Rat Gla-OC Competitive ELISA Kit and this kit were compared using 5 samples of rat serum and 2 samples of rat ascites.

		Rat Gla-OC HS Kit (Cat. #MK126)	Rat Gla-OC Competitive EIA Kit (Cat. #MK121; discontinued)
Dilution		25-Fold	Stock (no dilution)
Concentration (Stock solution-equivalent)		ng/ml	ng/ml
Serum	No. 12	369	400
	No. 13	353	1100
	No. 15	294	316
	No. 16	137	426
	No. 17	84	710
Ascites	No. 1	102	216
	No. 2	116	368

Result:
The results of the two kits were nearly equivalent about some samples and greatly different about others. This may be attributable to the difference in molecular species of Gla-osteocalcin in the samples.

9. Correlation with another company's kit

This kit and another company' s kit were used concurrently to assay 19 samples of rat serum. The kit from Biomedical Technologies (BTI) measures the amount of both forms of osteocalcin (Gla and Glu).



Result:
Nearly positive correlation was observed.

10. Effects of Sample Hemolysis

Blood collected from individual 4-week-old rats was divided in half. The first half was promptly centrifuged and then serum was collected. The second half was passaged through an injection needle a few times to induce hemolysis and then centrigued to collect the hemolysed serum. All samples were assayed simultaneously. Serially-diluted solutions were prepared (10, 20, 40, and 80 times) and assayed in order to determine the optimal dilution ratio. Additionally, measurement with a Rat Glu-Osteocalcin High Sensitive EIA Kit (Cat. #MK146) was carried out simultaneously in order to investigate whether hemolysis had any effect on the two kits.

The results for the 40 times diluted serum are shown for Cat. #MK126 and the results for the 20 times diluted serum are shown for Cat. #MK146.

Kit	MK126 Rat Gla-OC		MK146 Rat Glu-OC	
Dilution	40 times Dilution		20 times Dilution	
Sample	Normal Serum	Hemolytic Serum	Normal Serum	Hemolytic Serum
	Serum Concentration Converted Value (ng/ml)			
Rat 1	448.9	158.5	56.1	15.8
Rat 2	247.4	45.0	38.4	3.9
Rat 3	331.7	17.0	40.5	Below Sensitivity
Rat 4	549.0	9.8	79.5	Below Sensitivity

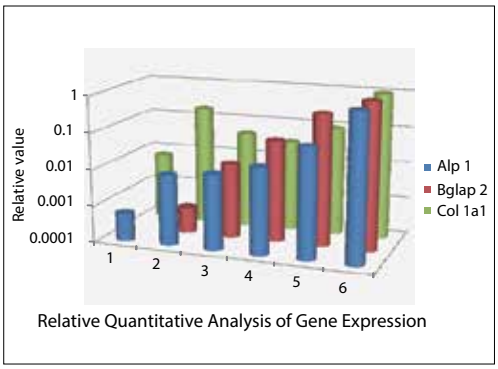
Results:
A tendency for hemolysis to cause both Gla-type and Glu-type osteocalcin measurements to be extremely low was observed in all individuals.

It is recommended that hemolytic serum be excluded from measurement or that the effects of hemolysis on the assay be taken into consideration.

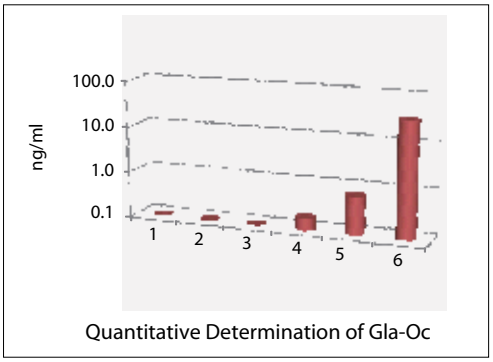
IX. Experimental Examples

1. Monitoring Gla-osteocalcin in cell culture supernatants

- 4-week-old rat's bone marrow cells (derived from a male SD rat (Cat. #MK433; discontinued)) were differentiated spontaneously or induced differentiation by addition of osteoblast-induce reagent. Levels of Gla-osteocalcin produced in the culture supernatants of bone marrow-derived cells were determined using this kit. Along with enzyme immunoassay, gene expression level was also monitored; total RNAs from cultured cells were prepared at the time of sample collection. Expression monitoring by intercalator-based real-time PCR at the mRNA level was performed on three genes; ALP (alkaline phosphatase), Bglap2 (bone Gla protein = osteocalcin), and Col1a1 (type-I collagen α1 chain).
- Culture supernatants collected at each differentiation stage contained fetal calf serum (bovine osteocalcin). However, since the solid-phase antibody in this kit is rat osteocalcin-specific, the supernatants were assayed directly using this kit.



Relative Quantitative Analysis of Gene Expression

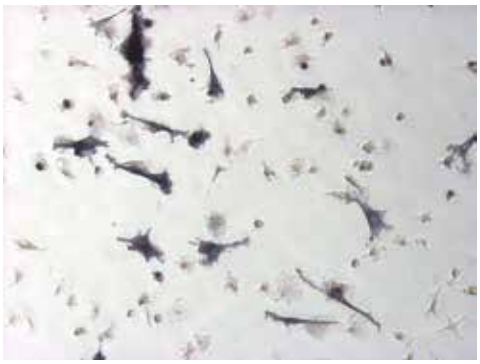


Quantitative Determination of Gla-Oc

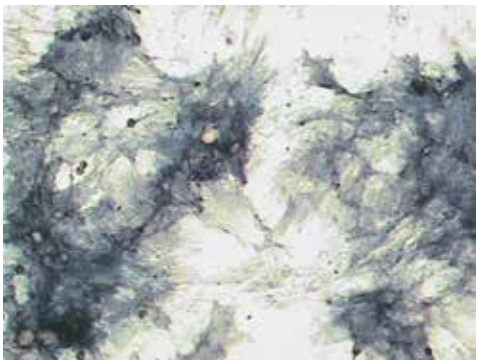
Reagents used: TB Green® *Premix Ex Taq*™ II (Perfect Real Time)
Apparatus: Thermal Cycler Dice™ Real Time System
Primer: Primers designed by the Perfect Real Time Support System (except Col1a1)

Assay Samples			Real time Assay mRNA Relative Quantitative Value			EIA Culture Supernatant (ng/ml)
Stage			Alp 1	Bglap 2	Col 1a1	Rat Gla-OC
1	BM4W day 4	Day 4 culture of bone-marrow cells	0.0006		0.007	0.000
2	BM4W OB-S1	Day 10 culture of bone-marrow cells (spontaneously differentiated)	0.009	0.0005	0.19	0.000
3	BM4W OB-L1	9-Day cultured bone-marrow cells + osteoblast induction Day 7	0.012	0.011	0.044	0.000
4	BM4W OB-L2	9-Day cultured bone-marrow cells + osteoblast induction Day 10	0.023	0.063	0.032	0.189
5	BM4W OB-L3	9-Day cultured bone-marrow cells + osteoblast induction Day 12	0.1	0.4	0.092	0.692
6	BM4W OB-L4	3-Day cultured bone-marrow cells + osteoblast induction Day 13	1.0	1.0	1.0	33.6

Results of Alkaline Phosphatase Strain



Stage 1 (Day 4 Culture)



Stage 5 (Day 10 Osteoblast Induction)

Alkaline phosphatase stain was performed using the TRACP & ALP double-stain Kit (Cat. #MK300).

2. Simultaneous Measurement of Gla-OC and Glu-OC in Rat Sera

Both types of osteocalcin (Gla- (active) type and Glu- (inactive) type) were monitored in the blood serum of young or aged rats over several weeks.

The values in the table are reduced concentrations multiplied by the dilution ratio.

	MK126					MK146				
	Rat Gla-OC EIA Kit					Rat Glu-OC EIA Kit				
Age (Weeks)	Dilution	Male No. 1	Male No. 2	Female No. 1	Female No. 2	Dilution	Male No. 1	Male No. 2	Female No. 1	Female No. 2
3	x 20	12.4	—	231.5	—	x 20	0.9	—	25.1	—
4	x 60	275.2	—	1389.2	—	x 20	33.6	—	62.0	—
5	x 60	277.6	—	375.3	—	x 20	40.1	—	48.2	—
6	x 60	445.0	380.6	143.7	372.5	x 20	71.8	52.2	20.1	43.1
7	x 60	235.3	357.0	178.3	494.6	x 20	40.0	52.5	26.0	56.5
8	x 60	351.4	356.2	530.1	840.2	x 20	64.5	48.0	61.4	74.5
9	x 60	180.5	272.1	301.9	154.0	x 20	28.5	43.4	44.0	23.2

Age (Weeks)	Dilution			Female No. R1	Female No. R2	Dilution			Female No. R1	Female No. R2
21	x 30			199.7	79.8	x 10			10.2	13.3
25	x 10			52.3	55.2	x 10			5.3	5.7
29	x 10			9.1	29.5	x 10			1.6	3.2
33	x 10			80.9	38.8	x 10			7.8	4.0

Units: ng/ml

Results:
Both Gla and Glu osteocalcin in sera tend to be higher than the aged rats suggesting activation of bone metabolic turnover.

X. Related Products

Rat Glu-Osteocalcin High Sensitive EIA Kit (Cat. #MK146)
Osteoblast differentiation reagent: Osteoblast-inducer Reagent (for animal cell) (Cat. #MK430)
TRACP & ALP double-stain Kit (Cat. #MK300)
TRACP & ALP Assay Kit (Cat. #MK301)
Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021)

XI. Precautions

1. Do not mix-use kits or reagents from different lots.
2. Do not expose (5) Substrate Solution (TMBZ) to strong light during storage or incubation. Avoid contact of Substrate Solution and Stop Solution with skin or mucous membranes. If these reagents come into contact with skin, wash thoroughly with water.
3. Use metal-free pipettes when handling (5) Substrate Solution (TMBZ).
4. Do not use (5) Substrate Solution (TMBZ) that has developed color.
5. Each reaction varies depending on time and temperature. Therefore, a new standard curve must be established for each assay.
6. Handle blood samples with great care.