Cat. # MK129

For Research Use

TaKaRa

Mouse Glu-Osteocalcin High Sensitive EIA Kit

Product Manual

v201607Da

Table of Contents

I. Description

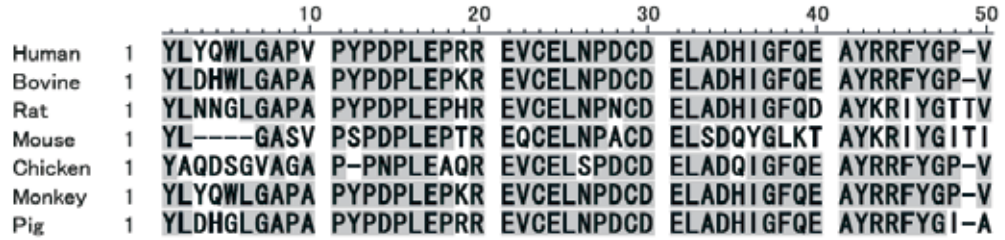
Osteocalcin (OC) is made up of 49 amino acids in humans (molecular weight of approximately 5,900 Da) and 46 amino acids in mice, and includes two to three γ -carboxyglutamate residues (Gla). Osteocalcin is a vitamin K-dependent calcium-binding non-collagen protein. Osteocalcin is only produced by osteoblasts and therefore is considered an osteoblast-specific marker. In particular, Gla-osteocalcin, which can bind with hydroxyapatite, is a marker of osteogenesis, while theGlu-osteocalcin, which cannot bind with hydroxyapatite, is a marker for bone resorption.

Unmodified Glu-type osteocalcin produced by osteoblasts is converted to active Gla-osteocalcin that can bind to the bone matrix through the conversion of glutamic acid to carboxyglutamate by vitamin K-dependent gamma-carboxylase. The Glu to Gla conversion of three residues within the osteocalcin molecule forms pockets that can trap calcium. The rate of conversion from Glu to Gla is not 100% in cells, and the amount of the two types of osteocalcin are usually equivalent. The two forms of the molecule are also present in the blood at levels that are thought to be related to bone metabolism. Recent findings suggest that Glu-type osteocalcin may also play an important role in the metabolism of sugar.

The Mouse Glu-Osteocalcin High Sensitive EIA Kit is an quantitative kit that enables specific and highly sensitive assay of decarboxylated osteocalcin from mouse bone tissue by enzymes present in osteoclasts and Glu-type osteocalcin (inactive osteocalcin) that has been produced by osteoblasts but has not undergone carboxylation.

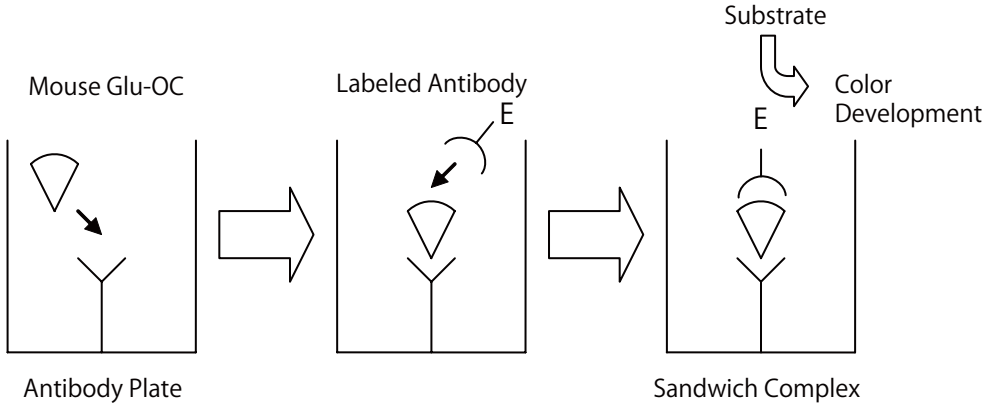
Because mouse osteocalcin has C terminal sequences that differ from those in humans, cattle, and other large animals, it is possible to measure mouse osteocalcin without cross-reaction with bovine antigens by using antibodies that recognize C-terminal epitopes. Therefore, it is possible to monitor osteoblastic cell differentiation from pluripotent cells such as mouse ES and iPS cells without interference from bovine serum included in the culture medium.

Bone turnover can also be analyzed by simultaneously measuring Gla-type and Glu-type osteocalcin. Gla-type osteocalcin can be evaluated using the Mouse Gla-Osteocalcin High Sensitive EIA Kit (Cat. #MK127), which includes a monoclonal antibody that specifically recognizes the Gla residues of osteocalcin.



Primary amino acid sequence alignment of osteocalcin from various animals.

II. Principle



III. Components

(1) Antibody Coated Microtiterplate Anti-Mouse OC Monoclonal Antibody Coated Plate (96 well: 8 well x12 strips)	1 plate
(2) Antibody-POD Conjugate(lyophilized) Peroxidase-Labeled anti-Glu-OC Monoclonal Antibody	for 11 ml
(3) Standard (lyophilized) Full-length synthetic mouse Glu-OC peptide 8 ng	for 1 ml
(4) Sample Diluent BlockAce containing PBS (with preservative)	11 ml x 2
(5) Substrate Solution (TMBZ) 3, 3′, 5, 5′ - Tetramethylbenzidine Solution	12 ml

IV. Materials Required but not Provided

1. Reagents

- Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021)
  Contains wash solution components (10X PBS, 50 ml x 5; Tween 20, 3 ml) and reaction stop solution (60 ml).
  ＊ This product is a peroxidase reaction stop solution that does not contain 1N sulfuric acid.
  Note : 1N sulfuric acid can also be used as the stop solution. Please handle 1N sulfuric acid with care.

2. Materials

- Pipette, micropipettes, and tips
- Microplate reader (that can assay absorptions up to 3.5 at 450 nm)

V. Storage

4℃

VI. Intended Use

- Quantitative determination of Glu-type osteocalcin (Mouse Glu-OC) in mouse biological samples.
- Quantitative determination of Glu-osteocalcin in supernatent from mouse osteoblast cultures.

Note : This kit is for research use only. It is not intended for diagnostic purposes.

VII. Protocol

1. Sample

- Suitable samples include mouse serum, plasma, peritoneal fluid, cell culture supernatant, and cell extract.
- Samples may be stored up to 12 hours at 2 - 10℃. If the assay will be performed longer than 12 hours after sample preparation, store samples frozen at -20℃.
- Use (4) Sample Diluent for dilution if necessary.
- The recommended dilution for mouse serum collected from 4 - 8-week-old mice is 3 to 4-fold. (Investigate the optimum dilution ratio before assaying a sample for the first time. A higher dilution may be needed for younger mice.)
- Because this product does not cross-react with bovine antigens, it can be used directly with cell culture media containing bovine serum.
- When using hemolyzed serum, the measured values will tend to be low.

2. Preparation of Reagents

- Antibody-Coated Microtiter plate

  Allow the (1) Anti-Mouse OC monoclonal antibody-coated plate to reach room temperature before use.

- POD-Labeled Antibody Solution

  Dissolve (2) Antibody-POD Conjugate with 11 ml of distilled water.

  After dissolution, the solution is stable for 1 week at 4℃. For longer storage, it is stable for 1 month at -20℃. However, only freeze and thaw once.

- Mouse Glu-OC standard solution

  Add 1 ml of distilled water to the lyophilized (3) Standard to reconstitute (8.0 ng/ml). Dilute the Standard with (4) Sample Diluent before use to prepare fresh serial dilutions of Standard at concentrations of 4.0, 2.0, 1.0, 0.5, 0.25, and 0.125 ng/ml. Use (4) Sample Diluent as the 0 ng/ml standard.

  The Mouse Glu-OC standard solution (8.0 ng/ml) is stable for up to1 week after preparation when stored 4℃ or for up to 1 month at -20℃. However, only freeze and thaw once.

- Substrate Solution

  Place (5) Substrate Solution (TMBZ) at 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 this coloration; make sure to avoid mixing with tap water.

  If the Substrate Solution will be used for several assays, divide it into aliquots of the required volume in advance.

- Reaction Stop Solution

  The stop solution included in Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021) is ready to use. This product is a peroxidase stop solution that does not contain sulfuric acid. Because the stop solution is a highly viscous fluid, mix well after adding with a plate mixer or similar device.

- PBS with 0.1% Tween 20 for Washing

  Dilute one bottle (50 ml) of 10X PBS from the Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021) with 450 ml of distilled water and add 500 μl of Tween 20. After mixing well, use this as the wash buffer (0.1% Tween 20/PBS).

3. Procedure

Assay samples in duplicate.
Bring 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 (1) Antibody Coated Microtiterplate quickly (within 5 minutes) using an 8-channel pipette or similar apparatus. To obtain highly reliable results, it is recommended that serial dilutions of the Standard Solution be placed 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 the reaction mixture, and wash 3 times with 0.1% Tween 20/PBS. Then add 100 μl of the POD-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, and wash 4 times with 0.1% Tween 20/PBS. 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 15 minutes. [Third reaction]

4. Add 100 μl of the stop solution＊ to each well in the same order as (5) Substrate Solution (TMBZ) was added to stop the reaction. Then mix well.
   ＊: The stop solution is a highly viscous fluid; mix well with a plate mixer or similar device after addition.

5. Set zero using distilled water as a control and measure absorbance at 450 nm. Color is stable for 1 hour after the reaction is stopped.

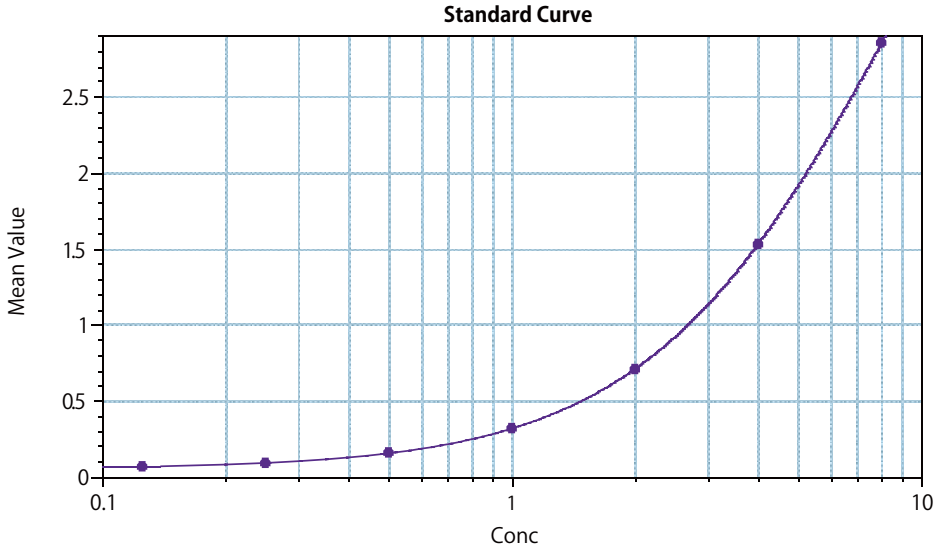
6. Plot a standard curve based on the results obtained from the Standard Solutions (with concentration as x-axis and absorbance as y-axis) and use it to determine the corresponding concentrations of mouse Glu-OC based on sample absorbance.

VIII. Performance

1. Standard Curve (Mouse Glu-Osteocalcin 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.

Limit of Detection: 0.25 ng/ml
Curve Fit : 4-Parameter



4-P Fit : $y = (A - D)/(1 + (x/C)^B) + D$:

A	B	C	D	R^2
0.0513	1.41	8.91	6.11	1

Mouse Glu-OC (ng/ml)	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0
Absorbance 450 nm	2.851	1.534	0.704	0.314	0.163	0.092	0.066	0.045

(Color development time : 15 min)

2. Reproducibility

<Intra-assay precision test (n=8)>

A reproducibility test was performed with 8 replicates using 3 concentration controls containing full-length mouse Glu-Osteocalcin peptides.

Specimen	Mean Value (ng/ml)	SD	CV (%)
Control A	4.124	0.159	3.9
Control B	1.861	0.040	2.2
Control C	0.948	0.025	2.6

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

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

Specimen	Mean Value (ng/ml)	SD	CV (%)
Control D	4.082	0.057	1.4
Control E	1.838	0.020	1.1
Control F	0.866	0.075	8.6

3. Recovery test

Samples containing different osteocalcin peptide concentrations were mixed equally with mouse serum samples. Sample Diluent was used to dilute the mouse serum samples and to prepare the peptide solutions. The amount of Glu-osteocalcin (ng/ml), as assayed using the EIA kit, for each mixture was compared with the theoretical value to determine the recovery rate.

When peptide solution was added to the 4X mouse serum, the average recovery rate was 111.7%. The recovery rate was higher when peptide was added to 2X mouse serum (average of 126%). When 2 types of peptide solution were mixed equally, the average recovery was 98.1%.

Differences in sample conditions may affect the recovery rate.

Sample A Mouse Serum (x 4)	Sample B Peptide Solution	Theoretical Value (A+B)/2	Measured Value	Recovery Rate (%)
2.150	4.139	3.145	3.523	112.0
2.150	1.903	2.027	2.454	121.1
2.150	0.894	1.522	1.852	121.7
2.231	4.139	3.185	3.277	102.9
2.231	1.903	2.067	2.243	108.5
2.231	0.894	1.563	1.766	113.0
1.730	4.139	2.935	3.340	113.8
1.730	1.903	1.817	2.034	111.9
1.730	0.894	1.312	1.321	100.7

Sample C Mouse Serum (x 2)	Sample B Peptide Solution	Theoretical Value (C+B)/2	Measured Value	Recovery Rate (%)
2.862	4.139	3.501	4.278	122.2
2.862	1.903	2.383	2.900	121.7
2.862	0.894	1.878	2.458	130.9
3.147	4.139	3.643	4.616	126.7
3.147	1.903	2.525	3.280	129.9

Sample D Peptide Solution	Sample E Peptide Solution	Theoretical Value (D+E)/2	Measured Value	Recovery Rate (%)
3.930	4.786	4.358	4.778	109.6
3.930	2.092	3.011	3.219	106.9
3.930	0.979	2.455	2.560	104.3
2.050	4.786	3.418	3.232	94.6
2.050	2.092	1.913	1.776	92.8
2.050	0.979	1.515	1.352	89.2
1.116	4.786	2.951	2.589	87.7
1.116	2.092	1.604	1.574	98.1
1.116	0.979	1.048	1.048	100.0

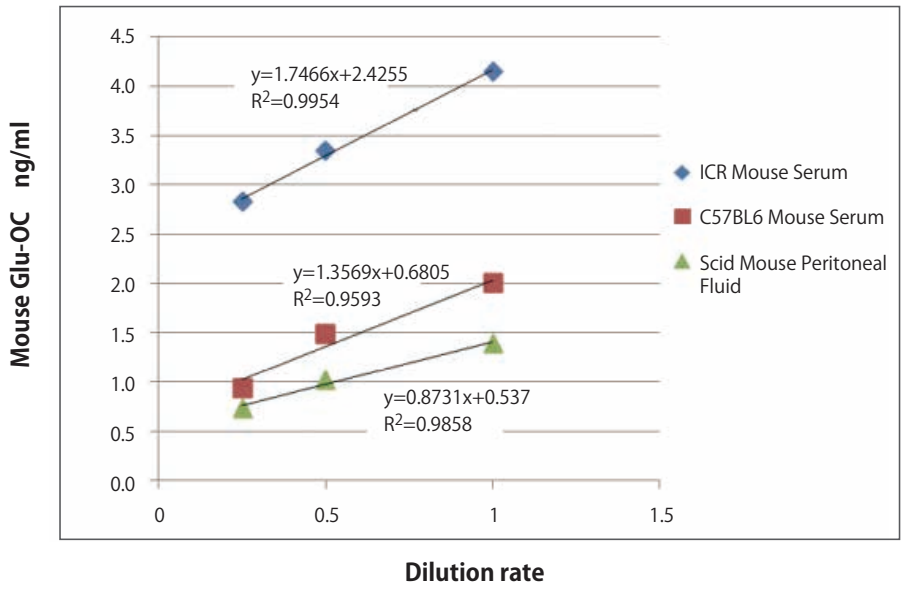
(ng/ml)

4. Linearity

Glu-osteocalcin measurement was performed on blood serum and peritoneal fluid samples from mice (undiluted, 2 fold, and 4 fold dilutions).
We recommend that serum and peritoneal fluid samples be diluted more than 2 fold so that linearity can be obtained.

Dilution Ratio	ICR Mouse Serum (8 - 9 Weeks)	C57BL6 Mouse Serum (8 - 9 Weeks)	Scid Mouse Peritoneal Fluid
x 1	4.155	1.997	1.395
x 2	3.350	1.480	1.019
x 4	2.828	0.939	0.725

(ng/ml)

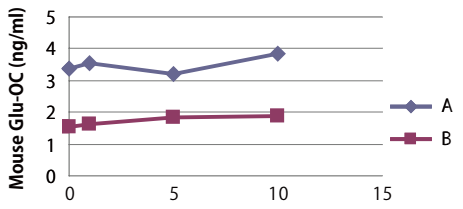


5. Effects of Coexisting Substances

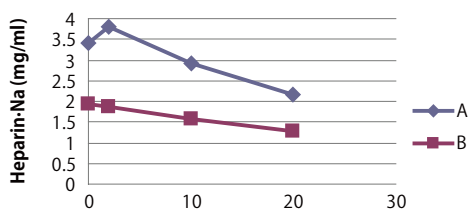
One volume of each coexisting substance was added to 9 volumes of osteocalcin standard solution (two concentrations, A and B), and the effects on the reaction were examined. In each graph, the final concentration of the coexisting substance is plotted on the x axis, and the Glu-type osteocalcin concentration (ng/ml) is plotted on they axis.

L-ascorbic acid and citric acid effected Glu-osteocalcin measurement, but no effects were observed when the pH was adjusted to a neutral range for substances such as sodium salts.

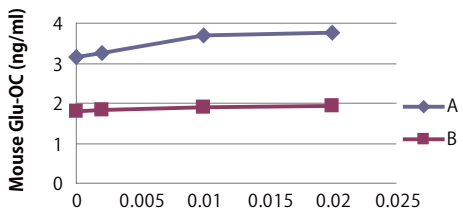
Heparin • Na



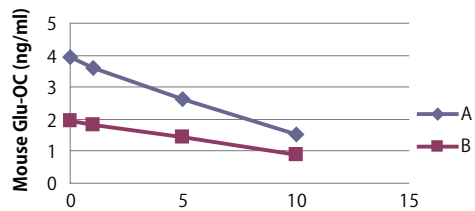
EDTA • 4Na



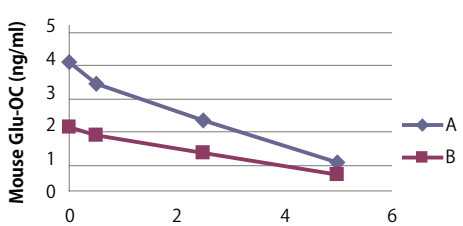
Bilirubin



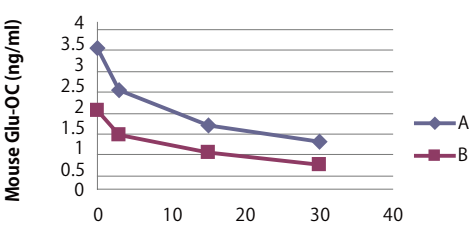
$CaCl_2 \cdot 2H_2O$



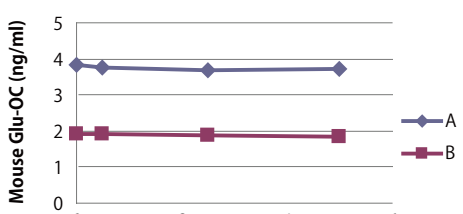
L-Ascorbic Acid



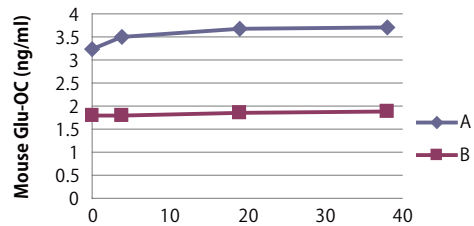
Citric Acid·2Na



L-Ascorbic Acid·Na



Citric Acid·3Na·2H_2O



IX. Experimental Example

1. Mouse cell culture medium

Primary osteoblasts isolated from the calvaria of newborn (3 days old or younger) mice were cultured, and Osteo-Inducer Reagent for Animal (Cat. #MK430) was used to induce osteoblast differentiation. The culture medium was collected over several days, and Glu-type and Gla-type osteocalcin were measured. In addition, calcification was monitored by staining with alizarin red.

- Culture media

  Basal Culture Medium: RPMI 1640 with 10% FCS, streptomycin and penicillin
  Osteoblast Differentiation Medium: Basal culture medium with Osteo-Inducer Reagent for Animal

- Cell number

  At time of inoculation: 1 x 10^5 Cells/well 24 well plate
  (Start of the experiment, 70% confluent; end of experiment,100% confluent.)

- Media volume

  3 ml per well of a 24 well plate

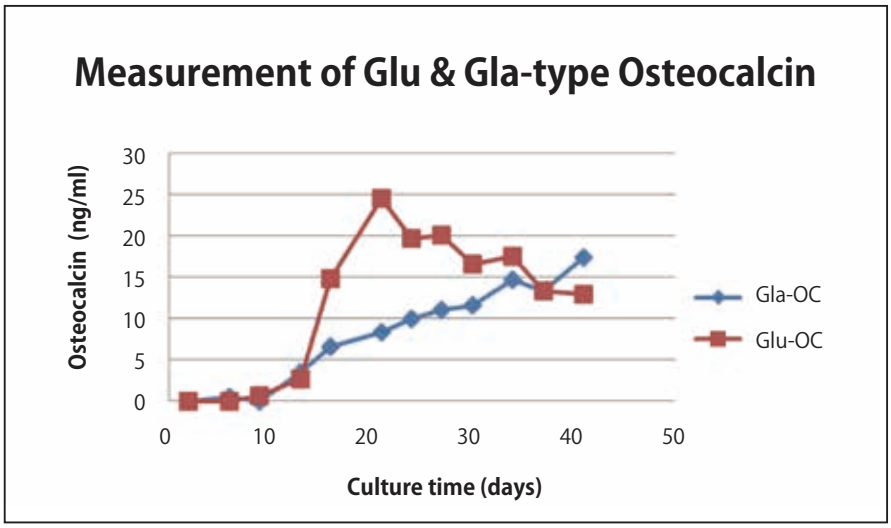
- Sample collection

  1 ml of culture supernatant was collected on the indicated days. An equal volume of fresh media was added at the same time.

- Measurement conditions

  For Glu-type osteocalcin, the Mouse Glu-Osteocalcin High Sensitive EIA Kit (Cat. #MK129) was used. For Gla-type Osteocalcin, the Mouse Gla-Osteocalcin High Sensitive EIA Kit (Cat. #MK127) was used. Samples were measured undiluted for both assays.

	Glu-OC (A450 nm)		Glu-OC (A450 nm)		Alizarin Red (A555 nm)	
Culture time (days)	Osteoblast Induction +	Osteoblast Induction −	Osteoblast Induction +	Osteoblast Induction −	Osteoblast Induction +	Osteoblast Induction −
2	0.050	0.048	0.048	0.045	0.039	0.038
6	0.047	0.059	0.057	0.042	0.064	0.060
9	0.069	0.053	0.048	0.043	0.068	0.068
13	0.170	0.045	0.067	0.045	0.090	0.084
16	1.382	0.057	0.102	0.044	0.073	0.070
21	2.343	0.048	0.129	0.043	0.564	0.107
24	1.882	0.048	0.161	0.042	1.264	0.081
27	1.917	0.049	0.180	0.043	2.649	0.076
30	1.561	0.090	0.192	0.044	3.672	0.071
34	1.660	0.048	0.267	0.042	3.712	0.099
37	1.241	0.051	0.235	0.042	3.634	0.093
41	1.169	0.048	0.342	0.046	3.702	0.112



<Results>

Both Gla-type and Glu-type osteocalcin were detected in the supernatant of cultured cells treated with osteoblast-inducing reagent. The amount of Glu-type osteocalcin peaked on day 21 and declined thereafter, whereas Gla-type osteocalcin increased throughout the timecourse.

For Gla-type osteocalcin, the incorporation into the bone matrix on the cell surface was observed through staining with mouse osteocalcin antibodies (data not shown), according to calcification of the cells.

X. Related Products

Mouse Gla-Osteocalcin High Sensitive EIA Kit (Cat. #MK127)
Human Gla-Osteocalcin High Sensitive EIA Kit (Cat. #MK128)
Gla-Type Osteocalcin (Gla-OC) EIA Kit (Cat. #MK111)
Rat Gla-Osteocalcin High Sensitive EIA Kit (Cat. #MK126)
Rat Glu-Osteocalcin High Sensitive EIA Kit (Cat. #MK146)
Pig Gla-Osteocalcin EIA Kit (Cat. #MK139)
Pig Glu-Osteocalcin EIA Kit (Cat. #MK149)
TRACP & ALP double-stain Kit (Cat. #MK300)
TRACP & ALP Assay Kit (Cat. #MK301)
Osteoblast-Inducer Reagent (for animal cell) (Cat. #MK430)
Anti-Mouse Osteocalcin, Monoclonal (Clone R21C-01A) (Cat. #M188)
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 reagents to strong light during storage or incubation.
3. Use metal-free pipettes when handling (5) Substrate Solution (TMBZ) and the Stop Solution.
4. Avoid contact of (5) Substrate Solution (TMBZ) and Stop Solution with hands or mucous membranes. If these reagents come into contact with skin, wash thoroughly with water.
5. Do not use a (5) Substrate Solution (TMBZ) that has developed color.
6. Each reaction varies depending on time and temperature. Therefore, a new standard curve must be established for each assay.
7. Handle blood samples with great care.