Cat. # MK127

For Research Use

TaKaRa

Mouse Gla-Osteocalcin High Sensitive EIA Kit

Product Manual

v201607Da

Table of Contents

I. Description

Osteocalcin (OC) comprises 49 amino acids, including 2 to 3 γ-carboxyglutamate residues (Gla), and has a molecular weight of approximately 5,900. It is known as a vitamin K-dependent calcium-binding non-collagen protein. Osteocalcin is an osteoblast-specific marker as it is produced only by osteoblasts. The Gla-osteocalcin, in particular, is a marker of osteogenesis.

[Primary amino acid structure of osteocalcin in various animals]

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                    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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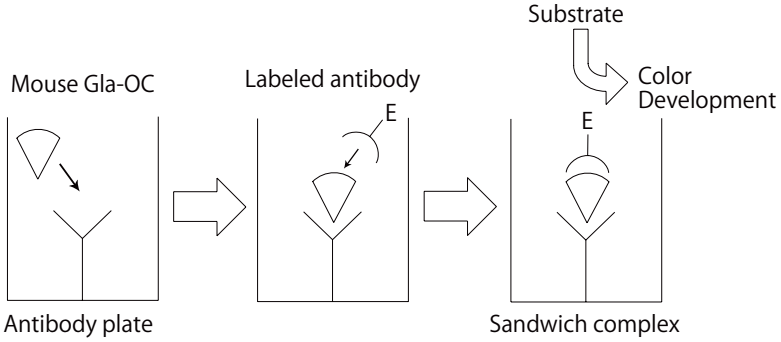
In bone formation, the dynamic osteogenesis in growing young animals is called “modeling” (new construction). In matured animals beyond the growing phase, bone morphology undergoes no apparent changes and remains stable, but a certain percentage of the bone are constantly being replaced. This process is called “remodeling” (reconstruction).

The Mouse Gla-Osteocalcin High Sensitive EIA Kit is an quantitative kit that enables specific and highly sensitive assay of mouse Gla-osteocalcin that exhibits a potential to osseointegration (active osteocalcin). The capture antibody (plate-bound antibody) is a plate-bound solid-phased rat monoclonal antibody that specifically recognizes the C-terminal region of mouse osteocalcin. It is paired with labeled antibody—a monoclonal antibody for detecting osteocalcin with Gla residues. Because mouse osteocalcin has C terminal region sequences that differ from those in humans, cattle and other large animals, it is possible to measure mouse osteocalcin without any cross-reaction with bovine antigens through capture of the antigen with antibodies recognizing a C-terminal epitope. Therefore, one can monitor the process of osteoblastic cell differentiation from pluripotent cells such as mouse ES and iPS cells without interference from bovine serum included in the culture medium.

Furthermore, this kit can be used to carry out high-sensitivity measurements on not only cell culture supernatents, but also samples of mouse blood and bodily fluids. For mice of approximately 8 weeks of age, measurement is possible using a sample dilution of 10 to 20-fold, enabling monitoring of Gla-type osteocalcin concentration even when it is only possible to collect a minimal volume of mouse serum.

II. Principle



III. Components

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

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

(3) Standard — for 1 ml
Full-length synthetic mouse Gla-osteocalcin peptide 16 ng (lyophilized)

(4) Sample Diluent — 11 ml×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

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

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, then store samples frozen at -20℃.
- Use (4) Sample Diluent for dilution if necessary.
- The recommended dilution for mouse serum collected from 8-week-old mice is 10 to 20-fold. (Investigate the optimum dilution ratio before assaying a sample for the first time. A higher rate of dilution may be needed for younger mice.)
- This product does not cross-react to human, bovine (including fetal bovine), rat, porcine, horse, chicken or guinea pig antigens.
- Because this product does not cross-react with bovine antigens, it can be used directly on supernatant of cell cultures propogated using media containing bovine serum.
- This product cross-reacts with rabbit antigens. It is therefore unsuitable for assaying samples of rabbit origin. We recommend Gla-Type Osteocalcin (Gla-OC) EIA Kit (Cat. #MK111) for assaying rabbit samples.

2. Preparation of Solutions

- Antibody Coated Microtiter plate

  Allow the (1) Anti-Mouse OC monoclonal antibody-coated 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.

- Mouse Gla-OC standard solution

  Add 1 ml of distilled water to the lyophilized (3) Standard to reconstitute it. (16.0 ng/ml).
  Dilute the Standard with (4) Sample Diluent before use to prepare fresh serial dilutions of Standard Solution at concentrations of 8.0, 4.0, 2.0, 1.0, 0.5, and 0.25 ng/ml. Use (4) Sample Diluent as the 0-concentration standard.

  The Mouse Gla-OC standard solution (16.0 ng/ml) is stable for up to1 week after preparation when stored 4℃, or for up to 1 month at -20℃. Once thawed, however, it may not be returned to frozen storage.

- 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 (1) Antibody Coated Microtiterplate quickly (within 5 minutes) 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 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, 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 minutes. [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 mouse Gla-OC based on the sample's absorbance.

Note:

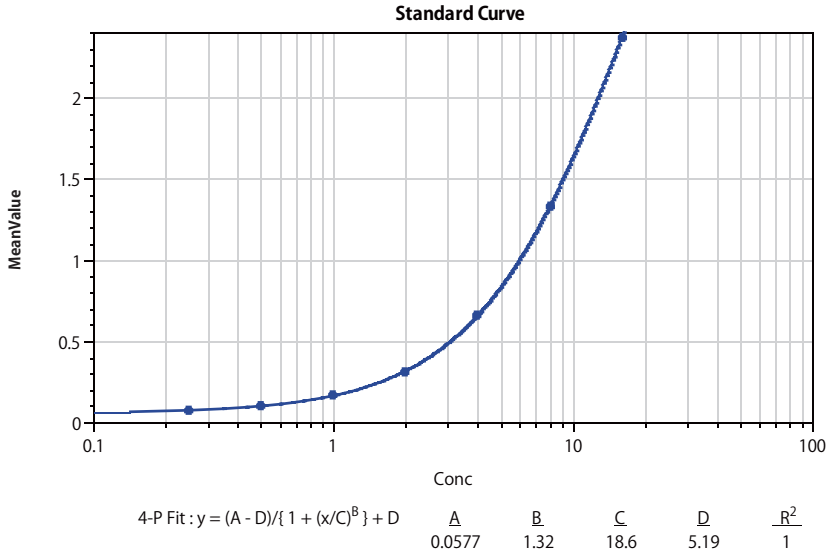
- Cover the plate with film or the like to prevent evaporation of solutions during reactions at room temperature or in an incubator.
- It is recommended that the Washing Buffer be completely discarded to get rid of the residual fluid.

VIII. Performance

1. Standard Curve (Mouse Gla-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: 0.5 ng/ml



Mouse Gla-OC concentration (ng/ml)	16.0	8.0	4.0	2.0	1.0	0.5	0.25	0
A_{450}	3.417	1.906	0.777	0.268	0.133	0.074	0.056	0.047

(Color development time: 15 minutes)

2. Reproducibility

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

A reproducibility test was performed with 8 replicates, using 3 different concentrations of mouse bone extract containing Mouse Gla-Osteocalcin.

Specimen	Mean (ng/ml)	SD	CV (%)
control A	8.136	0.429	5.3
control B	3.856	0.066	1.8
control C	1.732	0.033	1.9

<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 (ng/ml)	SD	CV (%)
control D	8.215	1.39	4.6
control E	3.940	0.11	2.7
control F	1.768	0.06	3.5

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 determine the recovery rate. The mean recovery rate was 103%.

Sample A	Sample B	Theoretical Value (A+B)/2	Assay Result	Recovery Rate (%)
8.62	15.82	12.22	13.01	106.4
8.62	8.35	8.49	9.04	106.6
8.62	4.46	6.54	6.70	102.4
8.62	2.12	5.37	5.55	103.3
8.62	1.10	4.86	4.90	100.7
4.07	15.82	9.95	10.77	108.3
4.07	8.35	6.21	6.70	107.9
4.07	4.46	4.26	4.17	97.7
4.07	2.12	3.09	2.95	95.4
4.07	1.10	2.58	2.64	102.1
1.83	15.82	8.83	9.71	110.0
1.83	8.35	5.09	5.12	100.6
1.83	4.46	3.15	3.19	101.4
1.83	2.12	1.98	2.07	105.0
1.83	1.10	1.47	1.46	99.4
1.83	0.58	1.21	1.22	101.5

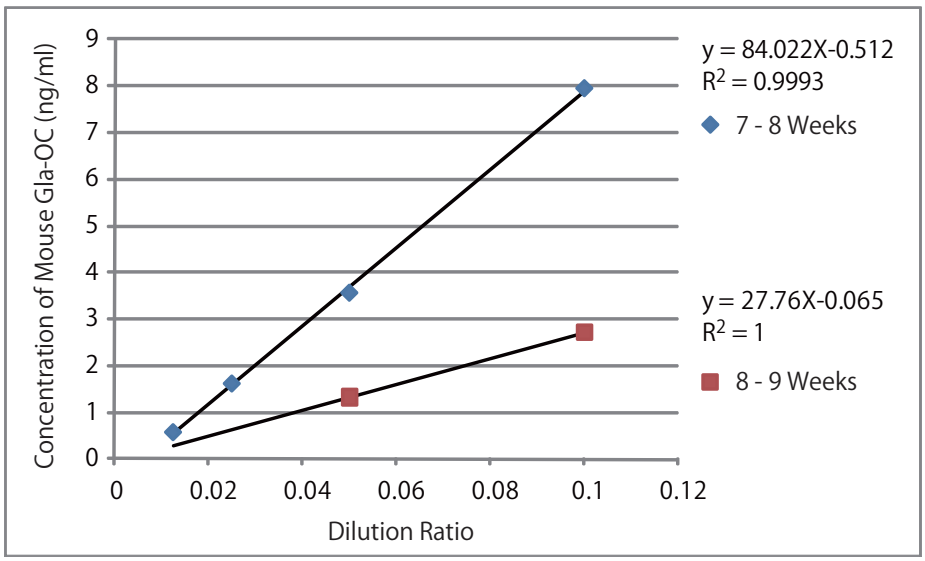
Unit: ng/ml

4. Linearity of Mouse Serum

Assay results blood serum samples from ICR mice (pooled serum, n = 15 mice) are presented below. It is preferable to assay 8 to 16-fold dilutions in the range of the standard curve for young mice, and undiluted to 5-fold dilutions for older mice.

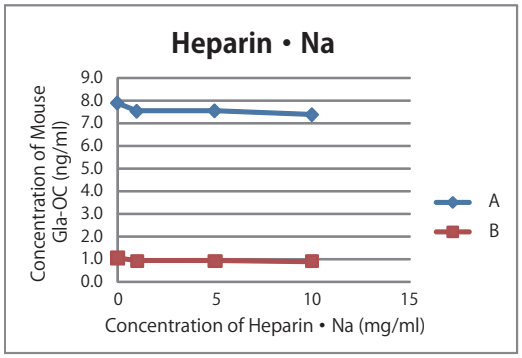
Dilution Ratio	7 - 8 week-old mice	8 - 9 week-old mice	Old Age
x 10	7.939	2.711	0.078
x 20	3.564	1.323	Below Limit of Quantitation
x 40	1.622	Below Limit of Quantitation	Below Limit of Quantitation
x 80	0.581	—	—

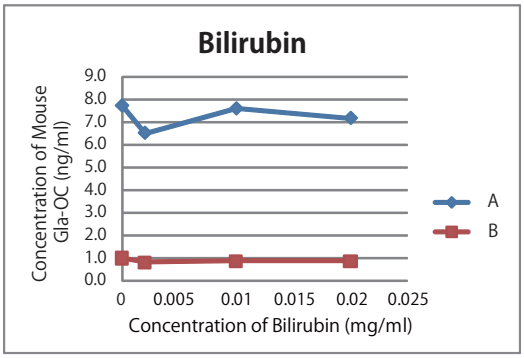
Units: ng/ml

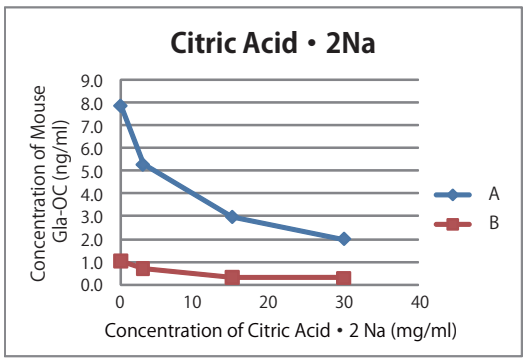


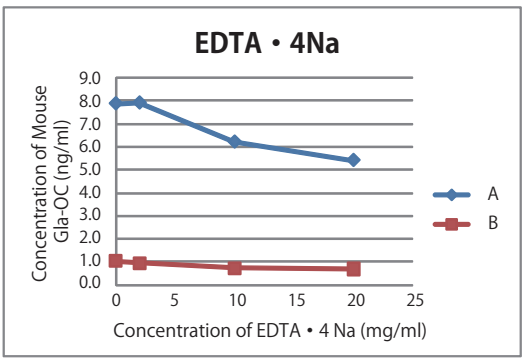
5. Effects of Coexisting Substances

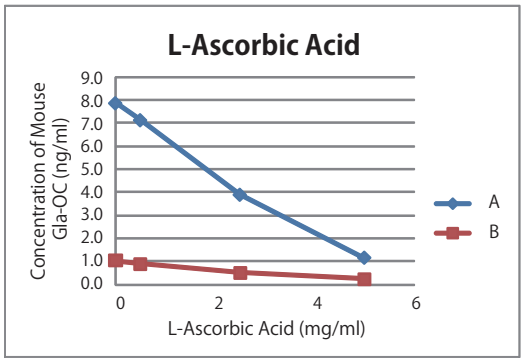
1 part by volume of the substance being tested was added to 9 parts by volume of osteocalcin Standard Solution (2 concentrations: A and B), and the effects on the reaction were examined. The final concentration of the test substance is shown on the horizontal axis of the graph. The concentration of Gla-osteocalcin measured is shown on the vertical axis. (Unit: ng/ml)

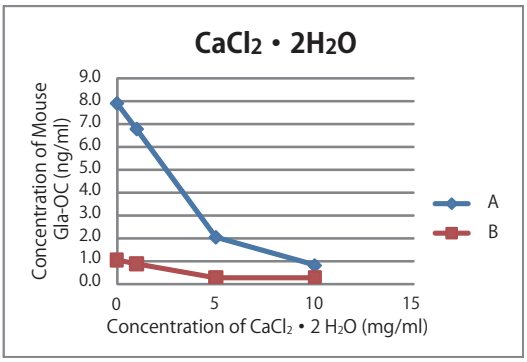












IX. Experimental Example

1. Assay of mouse cultured cells

We quantitatively monitored the individual serum Gla-osteocalcin levels in MC3T3E1 osteoblastic-like cell lines established from mouse skull bones.

Culture media:	DMEM, 10% FCS, with streptomycin and penicillin
Number of cells:	1 x 10^6 cells used for inoculation of one 10 cm plate. Growth monitored from 70% confluency to 100% confluency.
Medium Volume:	30 ml per10 cm plate
Sample collection:	500 μl supernatant samples were collected on the indicated days. Undiluted medium was used for the assay.

Culture (day)	Gla-OC concentration (ng/ml)
Blank (Medium)	0.000
1	0.559
5	0.51
10	0.863
20	3.500
22	4.364
24	3.856

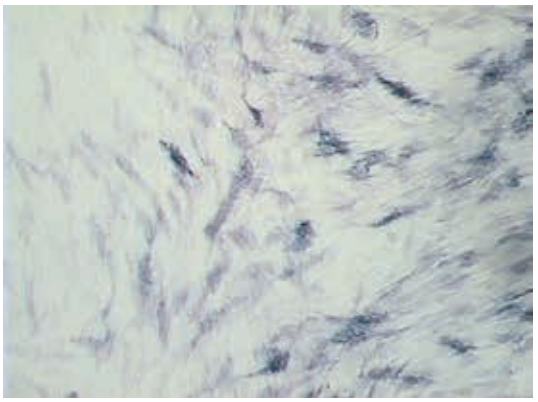
<Results>

In this experiment, cells were cultured in excess volume of the medium and monitoring was carried out continuously, without replacement of the medium.

The production of Gla-type osteocalcin in the culture supernatant was confirmed.

Alkaline phosphatase staining was carried out using the TRACP & ALP Double Stain Kit (Cat. #MK300) on day 0 and day 30 of culturing.

Day 0 of Culture



Day 30 of Culture

X. Related Products

Mouse Glu-Osteocalcin High Sensitive EIA Kit (Cat. #MK129)
Gla-Type Osteocalcin (Gla-OC) EIA Kit (Cat. #MK111)
Human Gla-Osteocalcin High Sensitive EIA Kit (Cat. #MK128)
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)
Anti-Osteocalcin (Mouse) (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.