Cat. # MK139

For Research Use

TaKaRa

Pig Gla-Osteocalcin 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 structures of osteocalcins of various species]

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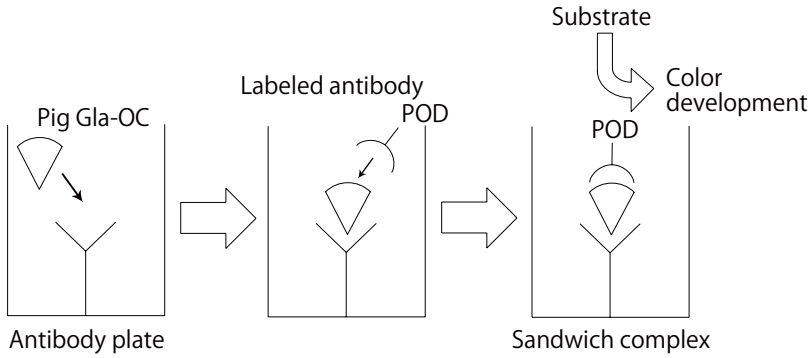
In bone formation, the dynamic osteogenesis in growing young animals is called “modeling” (new construction). In matured animals beyond the growing phase, bone morphology undergoes no apparent changes and remains stable, but a certain percentage of the bone are constantly being replaced. This process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

Efficacy assessments of osteoporosis drugs require the use of 2 types of animal models to allow evaluation for bone modeling and remodeling. Rodents, such as mouse and rat, are commonly used animal models to evaluate modeling, while monkey and miniature pig are used frequently to evaluate remodeling. These animal models are essential in the developments and efficacy assessments of potential therapeutic agents.

The Pig Gla-Osteocalcin EIA Kit is a quantitative kit that enables specific and highly sensitive assay of porcine Gla-osteocalcin that exhibits a potential to osseointegration (active osteocalcin). The capture antibody (plate-bound antibody) is a plate-bound solid-phased monoclonal antibody that specifically recognizes the Gla residue at position 17 on osteocalcin. It is paired with a labeled antibody—a monoclonal antibody for detecting porcine osteocalcin.

The concurrent measurement of undercarboxylated porcine osteocalcin (Glu-osteocalcin) may be achieved with the Pig Glu-Osteocalcin EIA Kit (Cat. #MK149) to monitor both bone formation and bone resorption based on a relative evaluation of Gla/Glu-osteocalcins.

II. Principle



III. Components

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

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

(3) Standard — for 1 ml
Full-length synthetic pig Gla-osteocalcin peptide 64 ng (lyophilized)

(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

Quantitative determination of Gla-type osteocalcin (Pig Gla-OC) in porcine biological samples.

VII. Protocol

1. Sample

- Suitable samples include porcine serum, plasma, ascite fluid, urine, and cell culture supernatant, 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 (4) Sample Diluent for dilution if necessary.
- The recommended dilution for porcine serum samples is 8 - 16-fold. (Investigate the optimum dilution ratio before assaying a sample for the first time.)
- As this product cross-reacts slightly with bovine antigens, any bovine-serum-supplemented medium in a cultured sample may interfere with the assay. Switch to a serum-free medium.
- This product cross-reacts slightly with rat antigens, resulting in low sensitivity. It is therefore unsuitable for assaying samples of rat origin. We recommend Rat Gla-Osteocalcin High Sensitive EIA Kit (Cat. #MK126) for assaying rat samples.
- This product does not react to mouse, rabbit, or human antigens.

2. Preparation of Solutions

- Antibody Coated Microtiter Plate
  Allow the (1) Anti-Gla 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.
- Pig Gla-OC standard solution
  Add 1 ml of distilled water to the lyophilized (3) Standard to reconstitute it (64.0 ng/ml). Dilute the Standard with (4) Sample Diluent before use to prepare fresh serial dilutions of Standard Solution at concentrations of 32.0, 16.0, 8.0, 4.0, 2.0, and 1.0 ng/ml. Use (4) Sample Diluent as the 0-concentration standard.

  The Pig Gla-OC standard solution (64.0 ng/ml) is stable for up to 1 week after preparation when stored at 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 Pig Gla-OC based on the sample's absorbance.

Note:

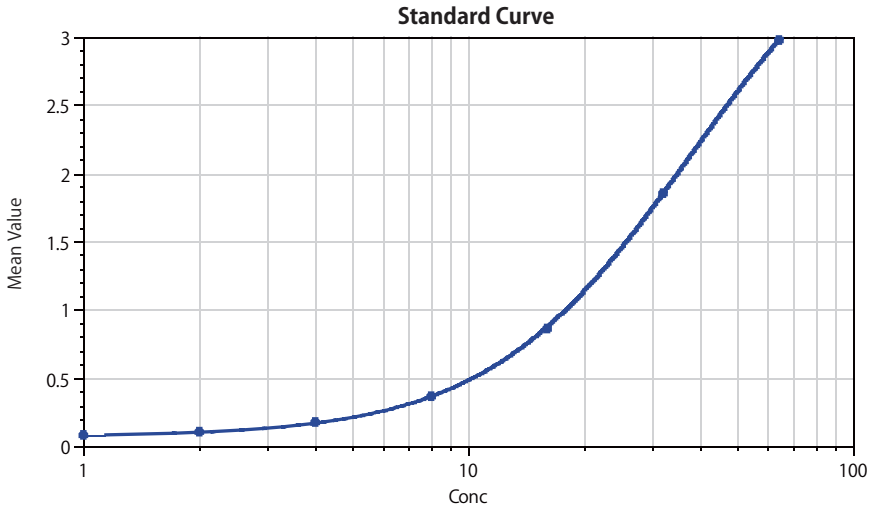
- Cover the plate with film or the like to prevent evaporation of solutions during reactions at room temperature or in an incubator.
- It is recommended that the Washing Buffer be completely discarded to get rid of the residual fluid.

VIII. Performance

1. Standard curve

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: 1.0 ng/ml



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

A	B	C	D	R^2
0.0699	1.65	37.6	4.18	1

Pig Gla-OC concentration (ng/ml)	64.0	32.0	16.0	8.0	4.0	2.0	1.0	0.0
A_{450}	2.975	1.858	0.864	0.371	0.174	0.105	0.082	0.060

(Color development time: 15 min)

2. Reproducibility

<Intra-assay precision test (n=6)>

A reproducibility test was performed with 6 replicates, using 3 different concentrations of porcine serum.

Sample	Mean (ng/ml)	SD	CV (%)
control A	51.4	0.66	1.3
control B	29.8	0.24	0.8
control C	19.7	0.57	2.9

<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)	SD	CV (%)
control D	26.6	0.21	0.8
control E	11.3	0.15	1.4
control F	6.5	0.18	2.8

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 102.8%.

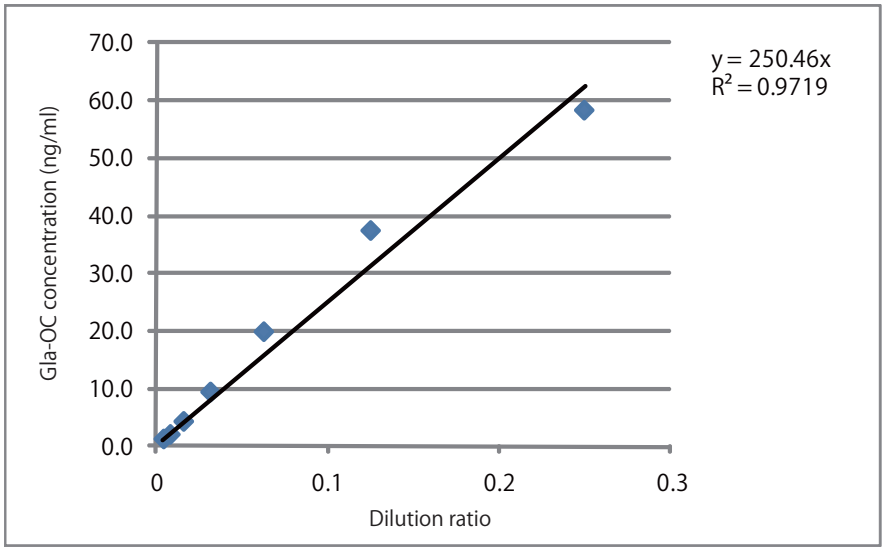
Sample A	Sample B	Theoretical Value (A+B)/2	Assay Result	Recovery Rate (%)
70.8	8.0	39.4	43.5	110.4
70.8	6.0	38.4	44.1	114.8
70.8	17.7	44.2	47.2	106.8
70.8	17.2	44.0	49.6	112.7
32.6	8.0	20.3	20.4	100.5
32.6	28.6	30.6	31.2	102.0
32.6	6.0	19.3	20.6	106.7
32.6	35.0	33.8	33.1	97.9
32.6	17.2	24.9	26.8	107.6
8.0	28.6	18.3	19.1	104.4
8.0	35.0	21.5	21.7	100.9
8.0	17.7	12.8	13.0	101.6
8.0	34.9	21.5	22.1	102.8
8.0	17.2	12.6	12.4	98.4
28.6	6.0	17.3	17.6	101.7
28.6	17.7	23.2	23.2	100.0
28.6	34.9	31.8	31.3	98.4
9.9	6.0	7.9	8.0	101.3
9.9	35.0	22.4	22.6	100.9
9.9	17.2	13.5	13.8	102.2
6.0	35.0	20.5	21.3	103.9
6.0	34.9	20.5	21.0	102.4
35.0	17.7	26.4	26.2	99.2
35.0	17.2	26.1	25.8	98.9
17.7	34.9	26.3	26.1	99.2
34.9	17.2	26.0	25.5	98.1

Unit: ng/ml

4. Linearity of Porcine Serum

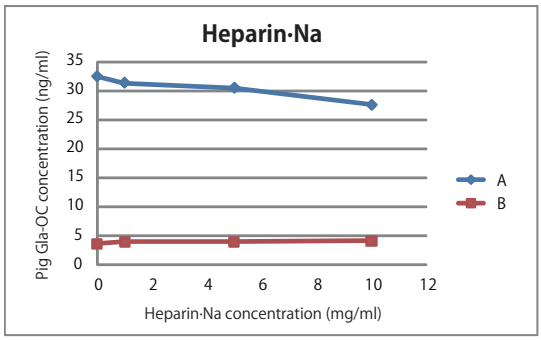
Assay results on a serum sample from an 8-month-old miniature pig are presented below. It is preferable to assay 8- or 16-fold dilutions in the range of the standard curve.

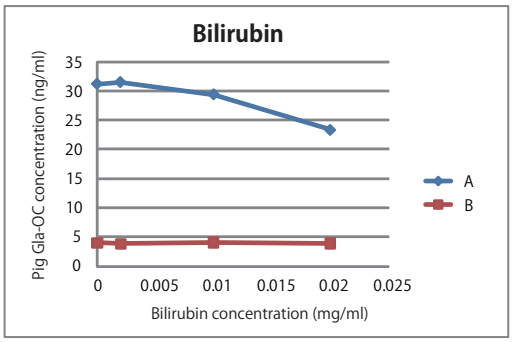
	Pig Gla-OC (ng/ml)
2X	70.9
4X	58.2
8X	37.4
16X	19.9
32X	9.5
64X	4.4
128X	2.2
256X	1.4

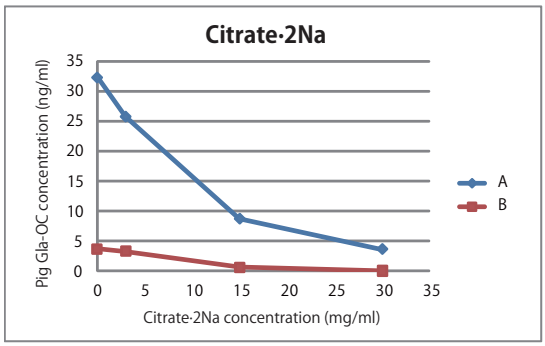


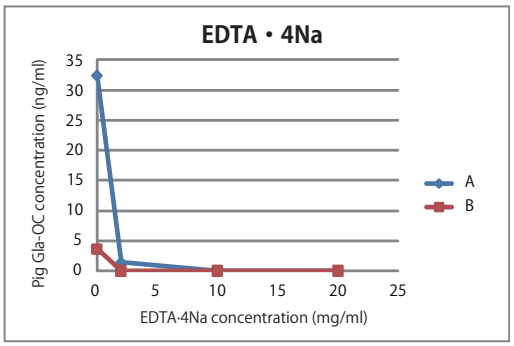
5. Effects of Coexisting Substances

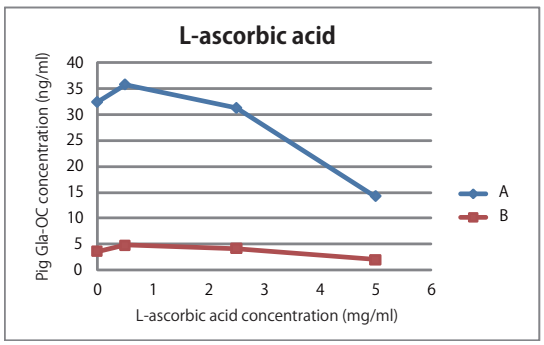
1 part by volume of the substance being tested was added to 9 parts by volume of osteocalcin Standard Solution (2 concentrations: A and B), and the effects on the reaction were examined. The final concentration of the test substance is shown on the horizontal axis of the graph. The concentration of Gla-osteocalcin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

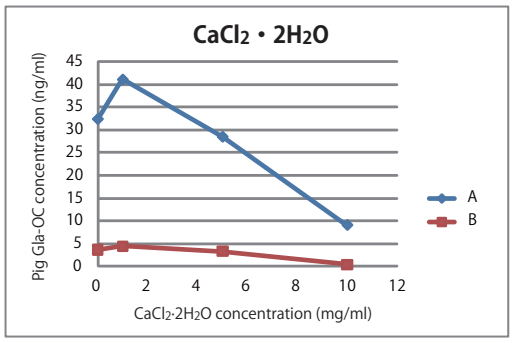












IX. Experimental Examples

1. Assay of miniature pig serum samples

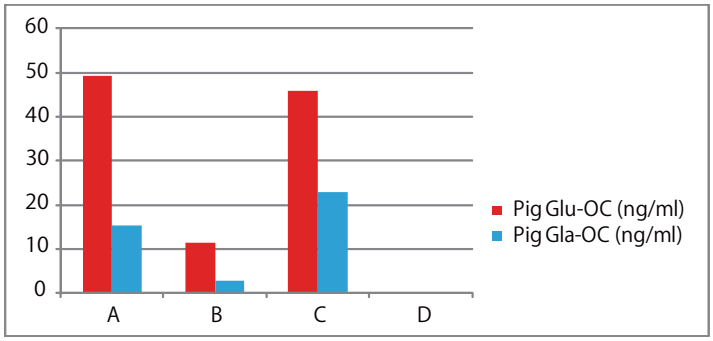
We monitored the individual serum Gla-osteocalcin levels in 4 miniature pigs (2 males, 2 females) aged 8 to 10 months old. Assayed samples were diluted 8- and 16-fold.

Miniature Pig		Dilution Ratio	
		8-Fold	16-Fold
Animal A (female)	8 months	34.3	16.9
	9 months	36.4	18.6
	10 months	25.6	13.2
Animal B (female)	8 months	21.4	10.4
	9 months	14.7	7.0
	10 months	14.4	7.3
Animal C (male)	8 months	44.0	23.4
	9 months	19.7	9.4
	10 months	21.0	10.8
Animal D (male)	8 months	46.6	23.8
	9 months	36.5	18.6
	10 months	30.8	15.7

[Result]
The levels of Gla-osteocalcin detected at 8, 9, and 10 months after birth declined gradually, suggesting the bone metabolic turnover had peaked over the growth phase and begun to settle down.

2. Monitoring processed food for components of pig bone-origin

We measured the Gla- and Glu-osteocalcin levels in pig bone extracts contained in processed food.
The powdered soups that came with 4 different types of instant food (A - D) were dissolved according to the recipes to prepare stock solutions, which were assayed for Gla- and Glu-osteocalcin levels.
With heating a part of their manufacturing process, the Gla-osteocalcin in these powdered soups may have become decarboxylated depending on the heating condition. However, the Gla-osteocalcin level in powdered soups A and C was detectable with this kit.



Gla-OC : Pig Gla-Osteocalcin EIA Kit (Cat. #MK139) was used for assay
Glu-OC : Pig Glu-Osteocalcin EIA Kit (Cat. #MK149) was used for assay

[Result]
The results demonstrated that this kit may be used to periodically monitor the content of pig-bone components during the manufacturing process of processed food.

X. Related Products

Pig Glu-Osteocalcin EIA Kit (Cat. #MK149)
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)
Human Gla-Osteocalcin High Sensitive EIA Kit (Cat. #MK128)
Anti-Osteocalcin (Bovine) (Cat. #M041- M044)
Anti-Osteocalcin (Human) (Cat. #M171, M184)
Anti-Osteocalcin (Mouse) (Cat. #M188)
Anti-Osteocalcin (Mouse), polyclonal (Cat. #M173)
Anti-Osteocalcin (Rat) (Cat. #M185 - M187)
Osteoblast Inducer Reagent (Cat. #MK430)
TRACP & ALP Assay Kit (Cat. #MK301)
TRACP & ALP Double Stain Kit (Cat. #MK300)
Anti-Rat Bone Specific Alkaline Phosphatase, Polyclonal (Cat. #M190)
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.