Cat. # MK111

For Research Use

TaKaRa

Gla-type Osteocalcin (Gla - OC) EIA Kit

Product Manual

v201804Da

Table of contents

I. Discription

Osteocalcin (OC), also known as bone γ -carboxylglutamic acid protein, is a vitamin K-dependent Ca^{2+}binding protein of molecular weight 5,900. It carries three carboxylated glutamic acid residues (Gla) at positions 17, 21, and 24 [1)] which are known to mediate strong binding of OC to hydroxyapatite. OC constitutes about 15% of the non-collagenous bone matrix proteins and is produced exclusively in osteoblasts and its dental counterpart, the odontoblast.[2)] Because of this tissue-specific expression, the level of OC could be considered as an indicator of the overall activity of cells operating in bone formation. Thus it could be suggested that when there is increased bone formation, the serum OC concentration will also be increased.[3)] Indeed, in clinical studies there is indication that aberrant levels of circulating OC reflect the occurrence of bone diseases.[4 - 7)]

Measurements of OC in serum samples are usually performed by competition immunoassays,[4)] however, these methods cannot distinguish between carboxylated and decarboxylated types of OC. The Gla-OC EIA Kit utilizes a novel set of monoclonal antibodies highly reactive to the carboxylated-type of osteocalcin (Gla-OC) and less reactive to the decarboxylated form, thus enabling a selective quantification of Gla-OC in biological fluids.[8)] The vitamin K-dependent calcium-binding properties of plasma proteins are usually dependent on the Gla residues. Calcium binding is generally necessary for biological activities such as activation of the blood coagulation cascade. In OC, the Gla residues are indeed necessary for the formation of a high affinity mineral-protein complex. Thus, it is likely that Gla-OC is the active form, and that measurements of Gla-OC by this EIA system may provide better leads of clinical information than do the conventional assays that cannot differentiate between active and inactive forms of OC.

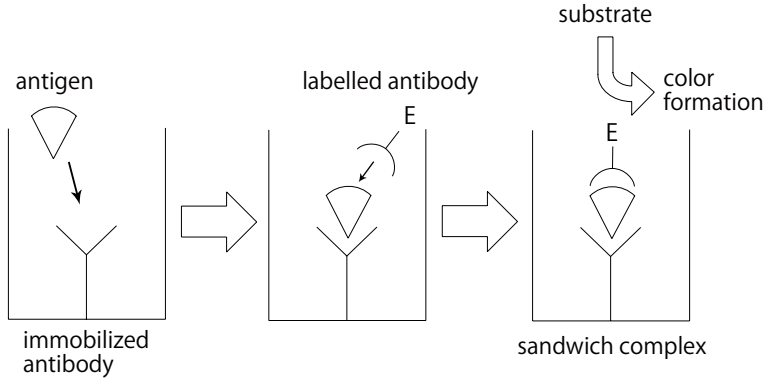
II. Intended Use

The Gla-type Osteocalcin EIA Kit is an *in vitro* enzyme immunoassay (EIA) kit for quantitative determination of human Gla-OC in serum, cultured cell extracts, cell culture supernatants, and other biological fluids.

This kit is for research use only. It is not for use in diagnostic or therapeutic procedures.

III. Principle

The Gla-OC EIA Kit is a solid phase EIA based on a sandwich method that utilizes two mouse monoclonal anti-Gla-OC antibodies to detect Gla-OC by two-step procedure. One of the mouse monoclonal anti-Gla-OC is immobilized onto the microtiter plate and blocked against non-specific binding. Samples and standards are incubated in the microtiter plate. The second step is to wash the plate and add the second anti-OC labelled with peroxidase (POD). During this incubation, Gla-OC is bound to anti-Gla-OC (solid phase) on one side and tagged on the other by POD-anti-OC. The reaction between POD and substrate (H_2O_2 and tetramethylbenzidine) results in colour development with intensities proportional to the amount of Gla-OC present in samples and standards. The amount of Gla-OC can be quantitated by measuring the absorbance using an EIA plate reader. Accurate sample concentrations of Gla-OC can be determined by comparing their specific absorbances with those obtained for the standards plotted on a standard curve.



IV. Components

(1) Antibody Coated Microtiterplate Anti-Gla-OC Monoclonal Antibody Coated Plate (96 wells: 8 wells x 12 Strips)	1 plate
(2) Antibody-POD Conjugate (lyophilized) Peroxidase-Labeled Anti-OC Monoclonal Antibody	for 11 ml
(3) Standard (lyophilized) Gla Osteocalcin Full-length Peptide 16 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

[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).

  Note : In this kit, Tween20 included in this puroduct (Cat. #MK021) is not used.
  - 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. Precautions

- Do not mix reagents from different kit lots.
- Do not use reagents beyond expiration date on label.
- In order to avoid reagent contamination, use disposable pipette tips and/or pipettes. Sodium azide inactivates POD. Solutions containing sodium azide should not be used in this assay.
- Do not expose Substrate Solution 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. Do not pipette by mouth. Do not smoke, eat, or drink in area where specimens or kit reagents are handled. All blood fluids should be considered as potentially infectious.
- Avoid contact of Substrate Solution and Stop Solution with any metal surfaces. Disposable glassware or test tubes are recommended for handling the Substrate Solution. If non-disposable is used, it must be acid washed and thoroughly rinsed with distilled, deionized water.
- Do not use the Substrate Solution if its colour is changed to thick blue.

VI. Specimen Collection and Handling

- Serum is suitable for use in the assay, however , plasma, cell culture supernatant, or cell extract can also be used.
- Serum is recommended for use in the assay in the case of blood sample.
- EDTA plasma is not suitable for use in the assay.
- The antibody used in this kit cross react with bovine, canine, rabbit, sheep, goat, and monkey Gla-OC. The measurement may be disturbed when it is performed with samples that includes animal serum (Ex. Fetal Bovine Serum (FBS)). It is recommended to perform the measurement with the kit under serum-free condition.
  In case of using human derived cell culture supernatant or cell extract containing bovine serum, Human Gla-Osteocalcin High Sensitive EIA Kit (Cat. #MK128) is recommended for this purpose.
- Samples may be stored up to 12 hours at 2 - 10℃. If the length of time between sample collection and assay is to exceed 12 hours, samples should be stored frozen under -20℃. Excessive freeze-thaw cycles should be avoided.
- In case of using serum or plasma, dilute the samples by 2 - 3 folds with Sample Diluent (4) before the assay.

VII. Preparation of Solutions

Note : The following solutions should be prepared just before use.

Solution 1. Antibody-POD Conjugate Solution Dissolve the contents of (2) in 11 ml distilled water and mix gently followed by 10 minutes slowly rolling or occasional mixing, avoiding foam formation.

Solution 2. Standard Solution Rehydrate Standard (3) with 1 ml distilled water. Slowly roll for approximately 10 minutes or let vials to stand and sporadically mix gently.

The Standard Solution contains 16 ng Gla-OC/ml. Prepare a dilution series of 8, 4, 2, 1, 0.5, and 0.25 ng/ml by diluting the Standard Solution with Sample Diluent.

VIII. Stability of Solutions

Solution 1. The reconstituted lyophilisate is stable for 1 week at 4℃ and for 1 month when stored at -20℃. Do not repeat freeze-thaw cycles.

Solution 2. The reconstituted lyophilisate is stable for 1 month when stored at -20℃. Do not repeat freeze-thaw cycles.

IX. Procedure

Double determinations of all samples and standards should be performed.
All of the Kit's content should be brought to room temperature before use.
For thorough mixing, the microtiter plate can be gently agitated on a plate mixer or by mixing the plate sporadically by hand.

[Enzyme immunoassay]

1. *Sample incubation* : Pipette 100 µl each sample and standard (Solution 2) into each well. All sample should be dispensed into wells of the microtiter plate within 5 minutes. Mix, seal the microtiter plate (e.g. with a foil) and incubate 2 hours at room temperature (20 - 30℃). Do not incubate sample at 37℃. When sample is incubated at 37℃, antigen would be denaturalized (proteolysis).
2. Remove sample solution and wash the wells 3 times with ca. 400 µl of PBS. Between the separate washing steps, empty out the microtiter plate and vigorously tap onto paper towel, especially after the last washing.
3. *Antibody-POD conjugate incubation* : Pipette 100 µl of Antibody-POD Conjugate Solution (Solution 1) into each well, mix, seal the microtiter plate (e.g. with a foil) and incubate 1 hour at room temperature (20 - 30℃).
4. Remove the solution and wash the wells 4 times as described above (It is especially important after this step to thoroughly empty out the remaining fluid before adding the substrate).
5. *Substrate incubation* : Add 100 µl Substrate Solution (5) into each well and incubate at room temperature (20 - 30℃) for 15 minutes.
6. Add 100 µl Stop Solution into each well in same order as for substrate. Tap plate gently to mix.
7. Measure the absorbance at 450 nm with a plate reader. The absorbance should be read as soon as possible after the completion of the assay. It may be read up to 1 hour after addition of Stop Solution if wells are protected from light at room temperature. Use a well of water as reference in the absorbance.

   Note : It is important that Stop Solution is added to wells prior to reading at 450 nm. Addition of Stop Solution causes an increase in absorbance of the Substrate Solution and shift in absorbance spectrum.

X. Results

1. Standard curve
   - Record the absorbance at 450 nm for each standard well.
   - Average the duplicate values and record the averages.
   - Plot the absorbance (vertical axis) versus the Gla-OC concentration in ng/ml (horizontal axis) for the standards.

2. Samples
   - Record the absorbance at 450 nm for each sample well.
   - Average the duplicate values and record the averages.
   - Locate the average absorbance value on the vertical axis and follow a horizontal line intersecting the standard curve. At the point of intersection, read the Gla-OC concentration (ng/ml) from the horizontal axis.

XI. Performance Characteristics

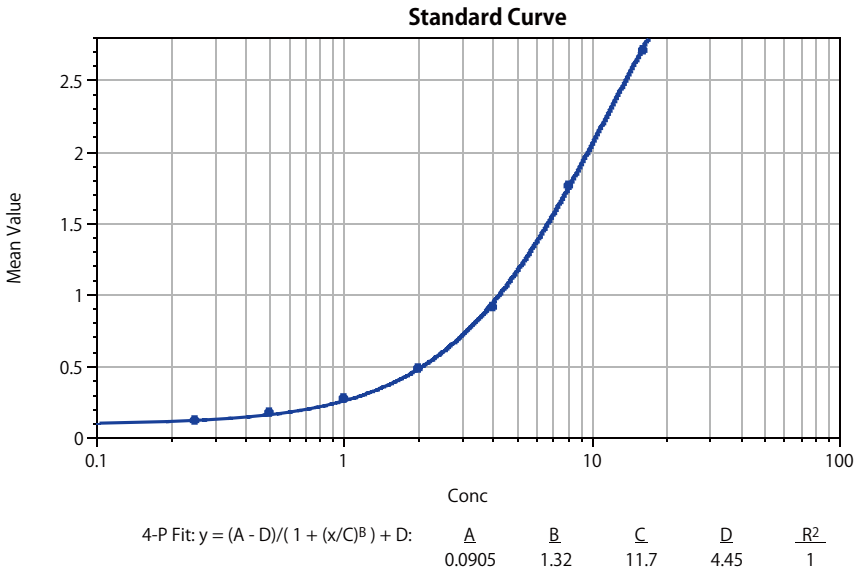
1. **Range of standard curve :** 0.5 - 16 ng/ml
2. **Specificity :**
   This kit specifically measures Gla-OC with no detectable cross reaction with the decarboxylated OC. This kit can be also used to measure bovine, canine, rabbit, goat, sheep, and monkey Gla-OC, but not to measure mouse Gla-OC.
   The application of this kit for quantitating Gla-OC from other sources has not been tested.
3. **Assay duration :** Three and a half hours after sample incubation
4. **Total assay capacity :** 96 assay
5. **Assay capacity for test samples :**
   If all assay wells (including standards and test samples) are run in duplicate, 40 test samples can be run in duplicate per kit.
6. **Test specimen type :**
   Serum, plasma, culture supernatants, cell extracts (Refer to "Specimen collection and handling", page 4.)
7. **Specimen volume required :**
   If each test sample is run in duplicate, approximately 220 μl (i.e., 100 μl per assay well plus ~10 μl for each sample transfer) is required. It is necessary to dilute blood sample containing high level Gla-OC about twice or three times.
8. **Limitation :**
   Since conditions may vary from assay to assay, a standard curve must be established for every run. Since cross contamination between reagents will invalidate the test, disposable pipette tips should be used.

   Thorough washing of the wells between incubations is required :
   1) Completely empty out the remaining fluid from the well before dispensing fresh wash solution.
   2) Use sufficient wash solution for each wash cycle (approximately 400 μl).
   3) Do not allow wells to sit uncovered for extended periods between incubation steps.

   Only samples with absorbance values falling within the range of the standard curve should be assigned a Gla-OC concentration from the curve.
9. **Notes :**
   According to the assay results using control serum, it could be possible to determine the concentration of antigen present in a biological. However, the measurement may be potentially disturbed by the unknown organic factors in serum samples in patients with specific diseases. Similarly, a specimen obtained from an apparent healthy subject might also be interrupted. When an antigen level in an unknown organic specimen is observed to be elevated as compared to the calibration range of the standard curve, it is recommended to dilute the specimens properly with the dilution solution included in the kit and assay them again in another run.

XII. Experimental Example

1. Standard curve



Gla-OC (ng/ml)	16.00	8.00	4.00	2.00	1.00	0.50	0.25	0.00
A_{450}	2.713	1.759	0.916	0.481	0.277	0.173	0.117	0.088

2. Reproducibility

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

Assay was carried out with 16 replicates of 3 samples containing different concentration of Gla-OC.

	Ave. (ng/ml)	S.D. (ng/ml)	CV (%)
Sample A	12.00	0.40	3.3
Sample B	1.48	0.04	3.0
Sample C	0.60	0.03	4.8

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

Assay to assay precision with one laboratory was evaluated in three independent experiments.

	Ave. (ng/ml)	S.D. (ng/ml)	CV (%)
Sample A	12.10	0.12	1.0
Sample B	1.49	0.01	0.7
Sample C	0.62	0.02	2.4

3. Recovery test

The recovery of Gla-OC was tested by adding two samples.

Sample A	Sample B	A＋B Measured	A＋B Calculated	Recovery (%)
11.10	0.00	5.40	5.55	97
11.10	11.10	11.10	11.10	100
11.10	5.41	8.43	8.25	102
11.10	2.28	7.08	6.69	106
11.10	1.09	6.30	6.09	103
11.10	0.68	5.63	5.89	96
5.41	0.00	2.47	2.70	91
5.41	5.41	5.41	5.41	100
5.41	2.28	3.97	3.84	103
5.41	1.09	3.07	3.25	94
5.41	0.68	2.67	3.04	88
2.28	0.00	1.11	1.14	97
2.28	2.28	2.50	2.28	110
2.28	1.09	1.65	1.68	98
2.28	0.68	1.41	1.48	95
1.09	0.00	0.64	0.55	117
1.09	1.09	1.11	1.09	102
1.09	0.68	0.86	0.89	97
0.68	0.00	0.50	0.34	148
0.68	0.68	0.65	0.68	95

(unit : ng/ml)

4. Epitope of the antibodies of this kit.

The first antibody : near osteocalcin, 17 position, γ -carboxylglutamic acid
Labeled antibody : osteocalcin, 4 - 9 amino acid residue

Intact Osteocalcin 1 .....17 ..... 21 .... 24.....49
Fragment Osteocalcin 1 .....17 ..... 21 .... 24..... 43

The above two forms having 17 position Gla residue will be detected in this ELISA.

5. Effect of hydroxyapatite treatment on serum Gla-OC value

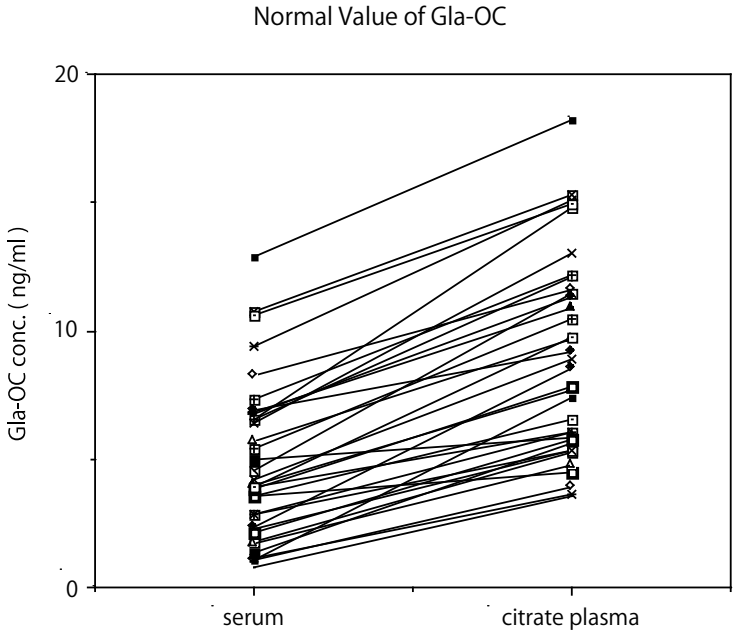
By treatment with hydroxyapatite, OC that binds to bone (active form) will be absorbed with hydroxyapatite. Active form of OC was recovered from hydroxyapatite by eluting with phosphate buffer solution. This ELISA system is useful for detection of active form of OC.

Sample No.	Serum Gla-OC	HAP treated serum Gla-OC	Gla-OC eluted with phosphate buffer
1	0.469	0.091	0.946
2	0.563	0.000	0.950
3	0.407	0.410	1.332
4	0.955	0.451	2.118
5	2.973	0.000	3.299
6	0.320	0.000	0.810
7	0.330	0.174	0.628
8	1.451	0.255	2.527
9	10.160	0.299	9.336
10	1.636	0.000	2.231
11	20.000	0.000	14.860
12	1.592	0.000	2.331
13	0.265	0.000	0.432
14	0.247	0.306	0.805
15	1.810	0.000	2.363
16	3.459	0.000	5.102
17	1.784	0.000	3.058
18	1.236	0.000	1.609
19	0.524	0.000	0.655
20	0.215	0.000	0.373
21	3.027	0.000	3.590
22	0.181	0.000	0.364
23	0.724	0.000	0.691
24	0.095	0.000	0.287
25	0.554	0.000	0.614
26	0.200	0.000	0.369
27	0.990	0.237	1.614
28	0.113	0.000	0.301

(unit : ng/ml)

6. Correlation of citrate plasma and serum value of Gla-OC

Gla-OC level in serum shows a tendency to be lower level than in citrate plasma.
Normal value of GlaOC

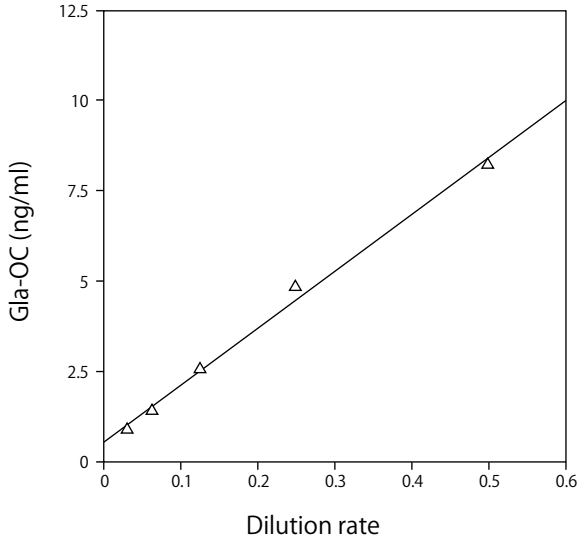


average	4.806 ng/ml	8.933 ng/ml
S.D.	3.052 ng/ml	3.896 ng/ml

n = 35 (age average 29)

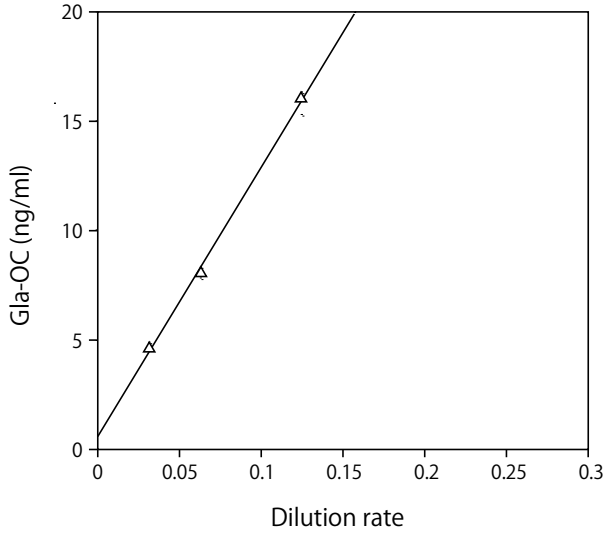
7. Dilution curves of human and rabbit serum samples

◆ Gla-OC dilution curve of normal human serum



y = 15.799X + 0.505
r = 0.998

◆ Gla-OC dilution curve of normal rabbit serum

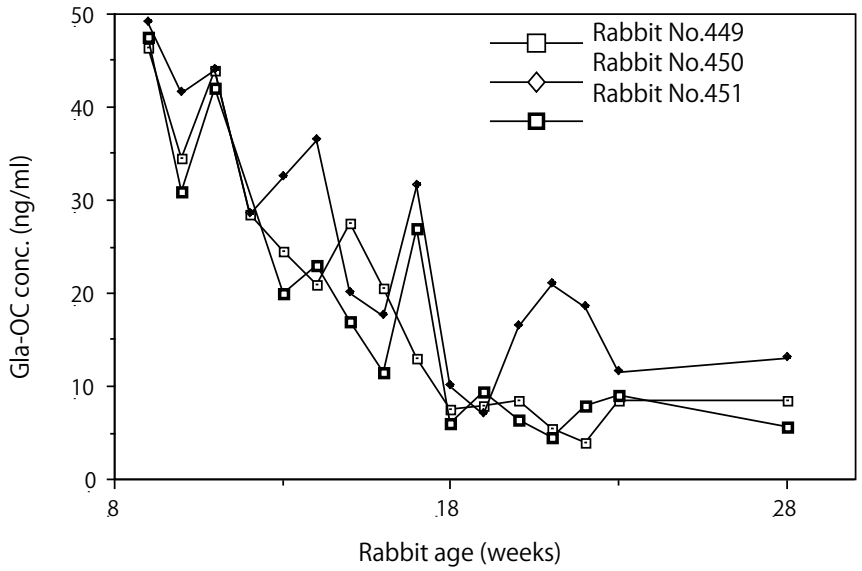


$y = 123.182X + 0.600$

8. Change of rabbit serum Gla-OC value in aging

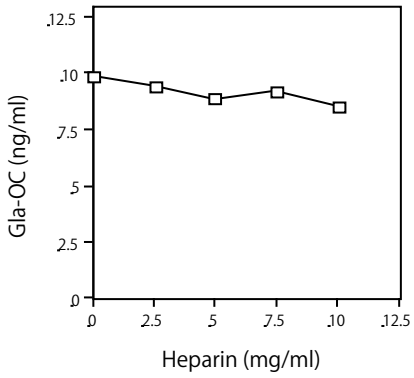
Rabbit (Japan White) serum samples were collected at intervals of one week from 9 weeks to 28 weeks. Following aging, serum Gla-OC was decrease remarkably.

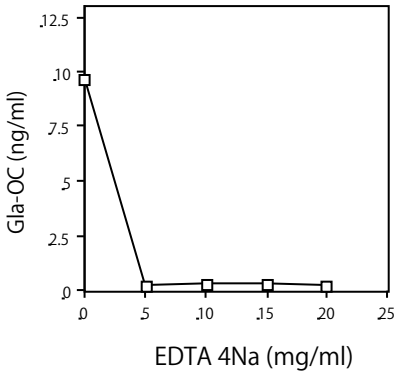
Alteration of serum Gla-OC

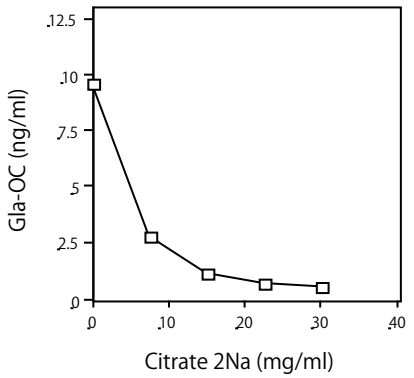


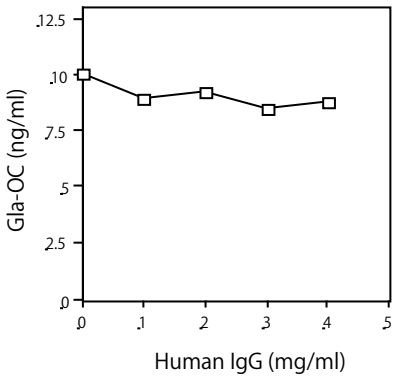
9. Influence of coexistence

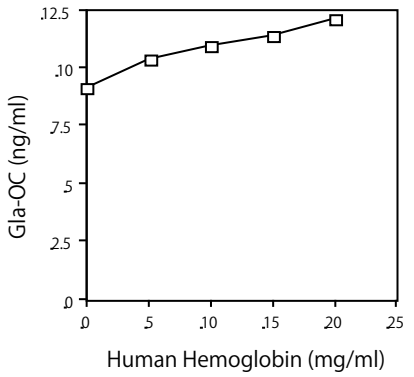
The volume ratio of sample to co-existing substance is 4:1. Co-existing substance is shown in its final concentration.

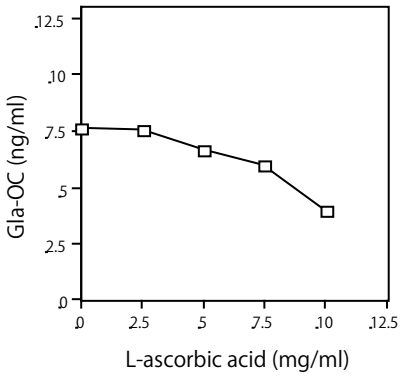


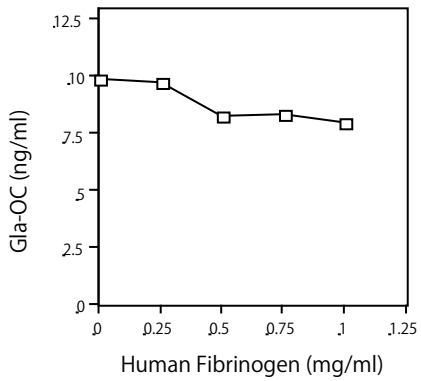


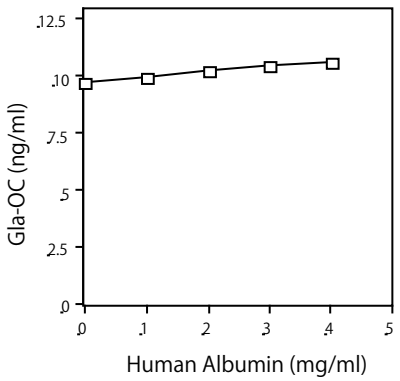


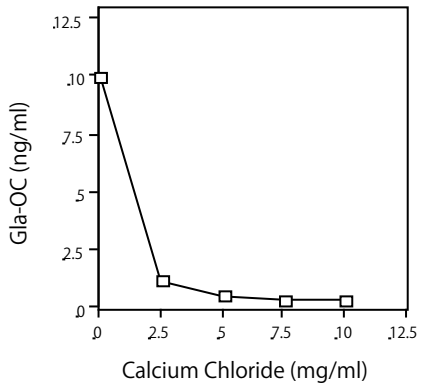


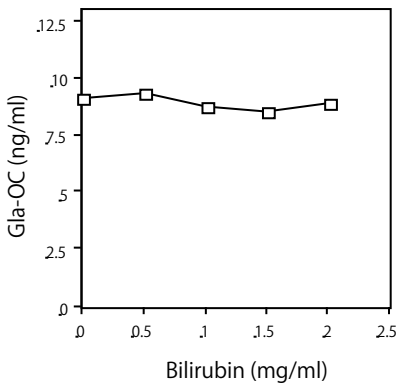






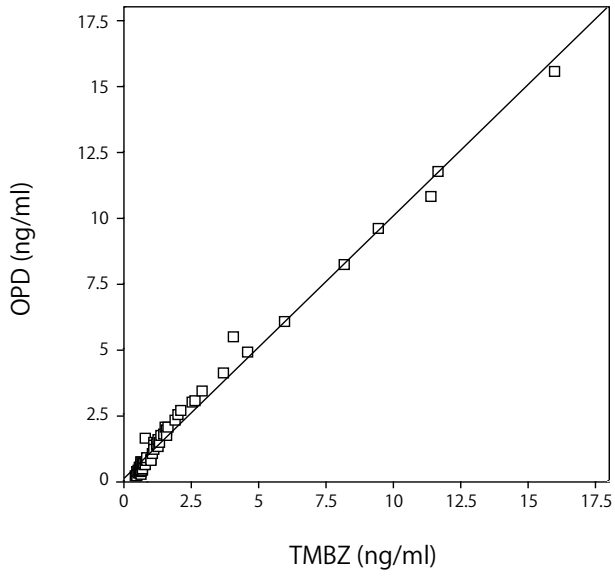






10. Correlation with the former kit (discontinued)

Correlation of O-phenylenediamine (OPD) to 3, 3', 5, 5'-tetramethylbenzidine (TMBZ) in Gla-type Osteocalcin EIA in using them as the substrate.



y = 0.997X + 0.098
r = 0.994

XIII. Storage and Stability

This kit is shipped at 4℃ and should be stored at 4℃ if not used. Under this condition, the kit is stable until the expiration date on label.

XIV. References

1) Poser J W, *et al*. *J Biol Chem.* (1980) **255**: 8685.
2) Price P A, *et al*. *Proc Natl Acad Sci USA*. (1976) **73**: 1147.
3) Deftos L J, *et al*. *Calcif Tissue Int.* (1982) **34**: 121.
4) Price P A, *et al*. *J Clin Invest*. (1980) **66**: 878.
5) Delmas P D, *et al*. *J Clin Invest.* (1983) **71**: 1316.
6) Malluche H M, *et al*. *Kidney Int*. (1984) **26**: 869.
7) Deftos L J, *et al*. *Clin Chem*. (1991) **37**: 1143.
8) Koyama N, *et al*. *J Immunol Meth*. (1991)**139**: 17.

XV. Protocol Summary

1. Add 100 μl of Standard or sample to appropriate wells, and incubate 2 hours at room temperature (20 - 30℃).
2. Remove sample solution and wash the wells 3 times with 400 μl of PBS.
3. Add 100 μl of Antibody-POD conjugate solution into wells and incubate at room temperature for 1 hour.
4. Aspirate solution from wells. Wash 4 times with 400 μl of PBS per wells, aspirating thoroughly between washes.
5. Add 100 μl of Substrate Solution to each well. Incubate 15 minutes at room temperature.
6. Add 100 μl of Stop Solution to all wells. Mix gently.
7. Read at 450 nm as soon as possible.