Storage and Stability

This kit is stable at 2 - 8°C, and can be used until the expiration date indicated on the kit box.

References

1. Poser, J.W. *et al.*(1980) *J. Biol. Chem.*, **255**, 8685.
2. Price, P.A. *et al.* (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 1147.
3. Deftos, L.J. *et al.* (1982) *Calcif. Tissue Int.* **34**, 121.
4. Price, P.A. *et al.* (1980) *J. Clin. Invest.* **66**, 878.
5. Delmas, P.D. *et al.* (1983) *J. Clin. Invest.* **71**, 1316.
6. Brown, J.P. *et al.* (1984) *Lancet*, **I**, 1091.
7. Malluche, H.M. *et al.* (1984) *Kidney Int.* **26**, 869.
8. Deftos, L.J. *et al.* (1991) *Clin. Chem.* **37**, 1143.
9. Koyama, N. *et al.* (1991). *J. Immunol. Meth.* **139**, 17.

Protocol summary

1. Add 100 µl of Standard or sample to appropriate wells, and incubate 2 hours at room temperature (20 - 25°C).
2. Remove sample solution and wash the wells 3 times with 400 µl of PBS.
3. Add 100 µl of Antibody-HRP 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.

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Zymed® Laboratories, Inc.

Gla-type Osteocalcin EIA KIT

An ELISA kit for the quantitative determination of Gla-type osteocalcin (Gla-OC)

Cat No. 99-0054
96 Wells

For research use only. Not for use in diagnostic or therapeutic procedures.

Table of contents

Introduction

Osteocalcin (OC), also known as bone γ-carboxylglutamic acid protein, is a vitamin K-dependent Ca^{2+}-binding protein with a molecular weight of 5900 Da. 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 [4-8].

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)

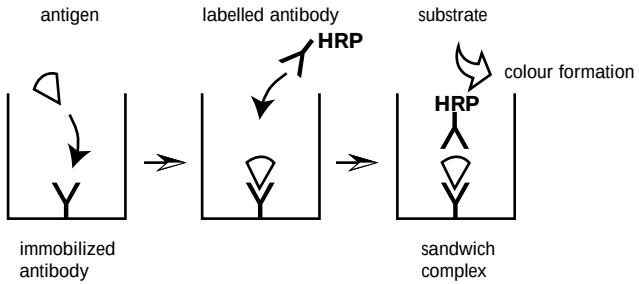
and less reactive to the decarboxylated form, thus enabling a selective quantification of Gla-OC in biological fluids (9). The vitamin K-dependent calcium-binding properties of plasma proteins are usually dependent on the Gla residues. Calcium binding is generally necessary for biological activities 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.

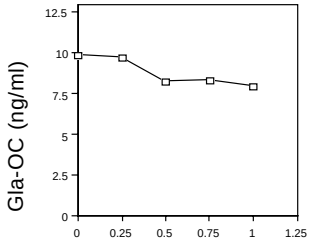
Intended use

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.

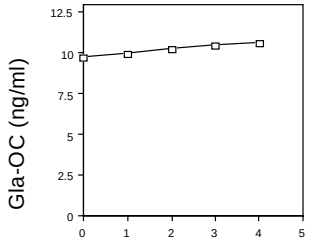
Principle

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 microtiterplate. The second step is to wash the plate and add the second anti-OC labelled with peroxidase (HRP). During this incubation, Gla-OC is bound to anti-Gla-OC (solid phase) on one side and tagged on the other by HRP-anti-OC. The reaction between HRP 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.

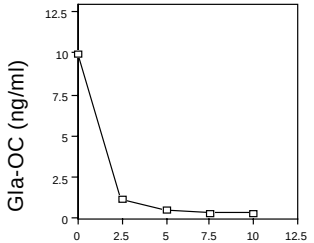




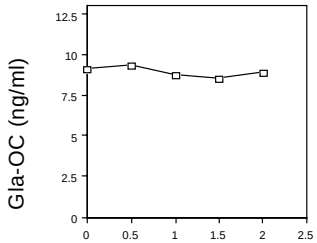
Human Fibrinogen (mg/ml)



Human Albumin (mg/ml)



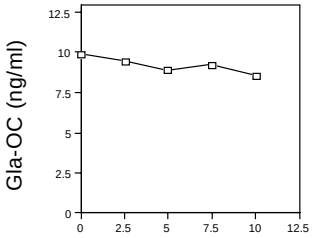
Calcium Chloride (mg/ml)



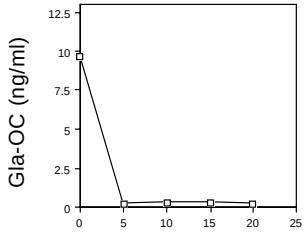
Bilirubin (mg/ml)

9. Influence of coexistence

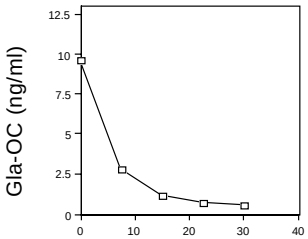
Co-existing substance is shown in its final concentration.



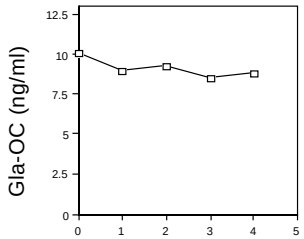
Heparin (mg/ml)



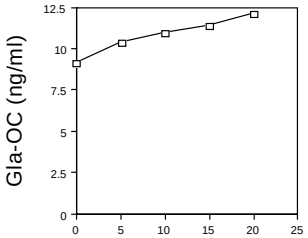
EDTA 4Na (mg/ml)



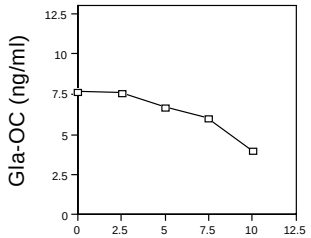
Citrate 2Na (mg/ml)



Human IgG (mg/ml)



Human Hemoglobin (mg/ml)



L-ascorbic acid (mg/ml)

Reagents and materials

Each Gla-OC EIA Kit includes reagents sufficient for 96 wells. The expiration date for the complete kit is stated on the outer box label and the recommended storage temperature is 2 - 8°C.

A. Materials provided

Plate 1. Antibody Coated Microtiterplate - 1 plate
The plate coated with murine monoclonal antibody to Gla-OC.
Store at 2 - 8°C.

Vial 2. Antibody-HRP Conjugate - 1 vial (11 ml x 1)
The vial contains lyophilized horseradish peroxidase (HRP) conjugated murine monoclonal antibody to OC. Store at 2 - 8°C. Avoid prolonged exposure to light.

Vial 3. Standard - 1 vial (16 ng x 1)
The vial contains lyophilized Gla-OC.

Vial 4. Sample Diluent - 2 vials (11 ml x 2)
Each vial contains protein in a buffered solution. Use for Zero standard, and for dilution of the standard (vial 3) and samples which are above the calibration curve. Store at 2 - 8°C.

Vial 5. Substrate Solution - 1 vial (12 ml x 1)
Each vial contains hydrogen peroxide and tetramethylbenzidine in a buffered solution. Store at 2-8°C.

B. Materials required but not provided

1. Reagents
   - Washing Buffer: Phosphate-buffered Saline (PBS)
     (Dissolve 8.0 grams of NaCl, 0.2 grams of KCl, 2.9 grams of $Na_2HPO_4 12H_2O$ and 0.2 grams of KH_2PO_4 in 1000 ml of distilled water.)
   - Stop Solution : 1 N H_2SO_4

2. Materials
   - Precision pipettes with disposable tips: 20 and 100 µl micropipettes, 10 - 200 µl adjustable multiwell pipetter or 20 µl and 100 µl multiwell pipetters
   - Beakers, flasks, cylinders necessary for preparation of reagents
   - Disposable pipettes and test tubes
   - Microtiter plate reader for measurement of absorbance at 450 nm
   - Graph paper

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 HRP. 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 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 and Stop Solution with any metal surfaces.
- Do not use the Substrate solution if its colour is changed to blue.

Specimen collection and handling

Cell or tissue extract is suitable for use in the assay, however, serum, or cell culture supernatant can be also used. PBS containing 0.5% Triton X-100, and 2 mM Phenylmethylsulfonyl fluoride (pH7.2) should be used for preparation of cell extracts. Venous blood samples are collected aseptically. Remove the serum from the clot or red cells, respectively, soon after clotting and separation. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. Samples may be stored up to 24 hours at 4ºC. If the length of time between sample collection and assay is to exceed 24 hours, samples should be stored frozen under -20ºC for optimal results. Excessive freeze-thaw cycles should be avoided. Prior to assay, frozen samples should be brought to room temperature slowly, and gently mixed by hand. Do not thaw samples in a hot bath. Do not vortex or sharply agitate.

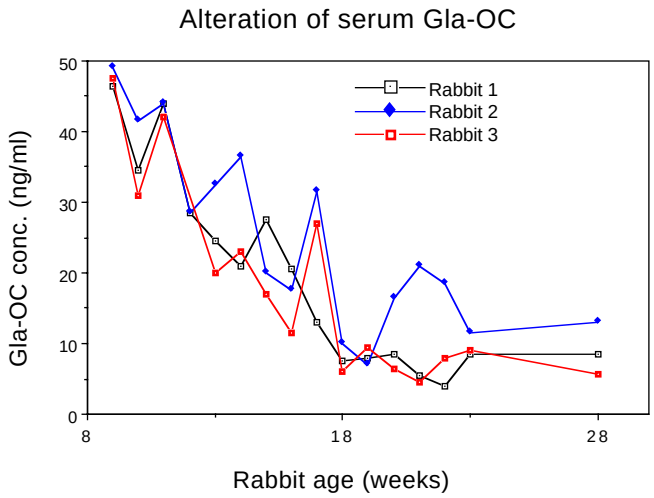
Preparation of solutions

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

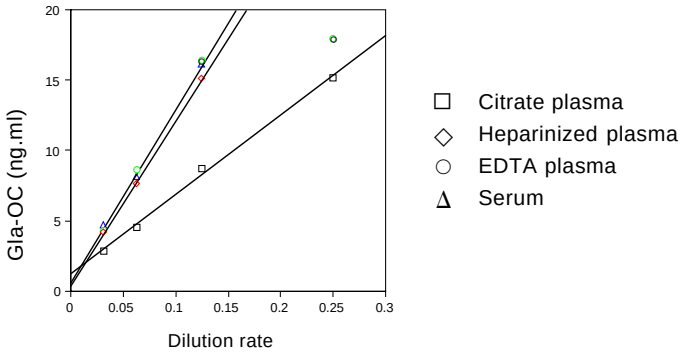
Solution 1.	Antibody-HRP Conjugate Solution Reconstitute Standard (Vial 2) with 11 ml distilled water. Slowly roll for approximately 10 min or let vials stand and gently mix at regular intervals. Avoid foam formation.
Solution 2.	Standard Solution Reconstitute Standard (Vial 3) with 1 ml distilled water. Slowly roll for approximately 10 min or let vials stand and gently mix at regular intervals. The standard solution contains 16 ng Gla-OC/ml.

8. Affect of aging on Gla-OC concentration in rabbits

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



Gla-OC dilution curve of normal rabbit sample



y = 56.463x + 1.211	Citrate plasma
y = 117.156x + 0.360	Heparinized plasma
Not fitting	EDTA plasma
y = 123.182x + 0.600	Serum

Stability of solutions

- Solution 1. The reconstituted stability is 1 week at 4°C .
- Solution 2. The reconstituted stability is 1 week at 4°C, and 1 month when stored at -20°C.

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 regularly by hand.

[Enzyme immunoassay]

- *Sample incubation*: Pipette 100 µl sample and standard (Solution 2) into the appropriate wells within 5 minutes. Mix, cover the microtiter plate (e.g. with foil) and incubate 2 hours at room temperature (20 - 25°C). Do not incubate serum at 37°C. In case serum is incubated at 37°C, antigen would be denaturalized (proteolysis).
- Remove sample solution and wash the wells 3 times with ~ 400 µl of PBS; between the washing steps empty out the microtiter plate and vigorously tap onto paper towl, especially after the last washing.
- *Antibody-HRP conjugate incubation*: Pipette 100 µl of Antibody-HRP Conjugate Solution (Solution 1) into one well, mix, cover the microtiter plate (e.g. with foil) and incubate 1 hour at room temperature (20 - 25°C).
- Remove sample 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).
- *Substrate incubation*: Add 100 µl Substrate Solution (vial 5) into each well and incubate at room temperature (20 - 25°C) for 15 min.
- Add 100 µl Stop Solution (1N H_2SO_4) into each well in the same order as for the substrate. Tap plate gently to mix.
- Measure the absorbance at 450 nm with a plate reader. The absorbance should be read as soon as possible after 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.

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.*

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 using log-log scale.

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.

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 also be used to measure bovine, canine, rabbit, porcine, goat, sheep, and chicken Gla-OC, but not 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 wells.
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:** plasma, serum, culture supernatants, cell extracts.
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 neccesary to dilute blood samples 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:

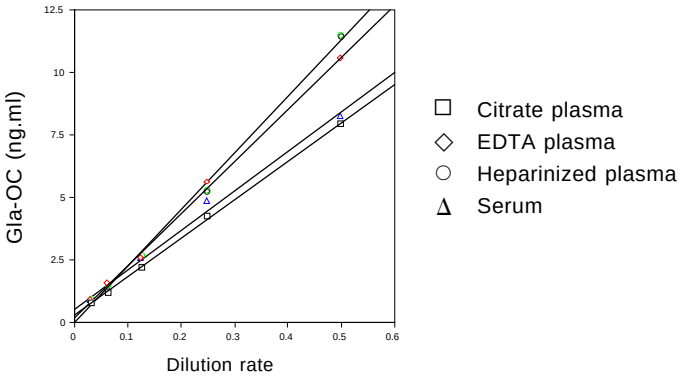
1) Completely empty out the remaining fluid from the well before dispensing fresh wash solution.
2) Use sufficient wash solution for each wash cycle (approximately 400 µl).
3) Do not allow wells to sit uncovered for extended periods between incubation steps.

Only samples with absorbance values falling within the range of the standard curve should be assigned a Gla-OC concentration from the curve.

7. Effect of anticoagulants

Effect of anticoagulants was evaluated by comparing the dilution curve of the samples which were simultaneously treated with different anticoagulants.
(Normal human sample and normal rabbit sample)

Gla-OC dilution curve of normal human sample

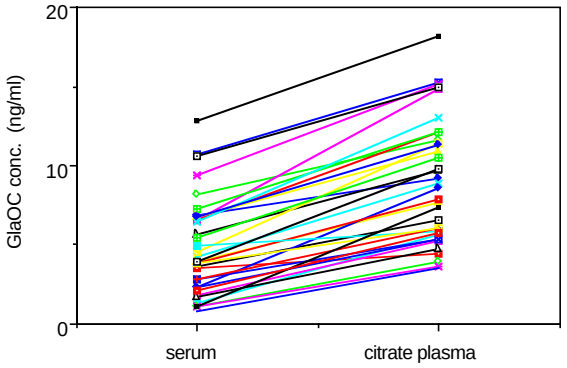


y = 15.400x + 0.274	r = 1.000	Citrate plasma
y = 20.813x + 0.182	r = 0.999	EDTA plasma
y = 22.663x - 0.013	r = 0.999	Heparinized plasma
y = 15.799x + 0.505	r = 0.998	Serum

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

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

Normal value of Gla-OC



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

(n=35, age average 29)

9. **Notes :** According to the assay results using control serum , it could be possible to determine the concentration of antigen present in a biological sample. However, the measurement may be potentially disturbed by the presence of unknown organic factors in serum samples from patients with specific diseases. Similarly, a specimen obtained from an apparently healthy subject might also be disturbed. 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 such specimens properly with the dilution solution included in the kit and assay them again in another run.

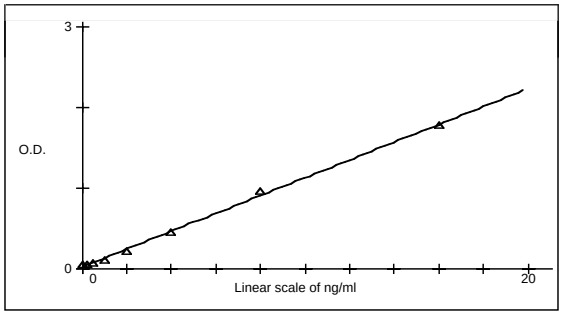
Basal data

1. Typical standard curve

Typical Standard Curve
(Do Not Use To Calculate Unknowns)

Curve Fit: Linear Corr. Coeff: 0.999
y = A + B * x

A = 0.0349 B = 0.110



Gla-OC (ng/ml)	16	8	4	2	1	0.5	0.25	0
A450	1.783	0.968	0.462	0.231	0.125	0.085	0.071	0.058

2. **Intra-assay precision** (n = 16)

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

	Avg. (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 (n = 3)

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

	Avg. (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 serum samples at five different level in various matrices.

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

4. Epitope of the antibodies of this kit.

The first antibody : near osteocalcin, 17 position, γ-carboxylgultamic 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 powder 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.

(unit: ng/ml)

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