Cat. # MK118

For Research Use

TaKaRa

Undercarboxylated Osteocalcin (Glu-OC) EIA Kit

Product Manual

v201804Da

Table of Contents

I. Description

Osteocalcin (OC), also known as bone gla protein (BGP), is a 49 amino acid vitamin K-dependent calcium binding protein with a molecular weight of 5.9 kDa. Carboxylated OC (Gla-OC), which is capable of binding calcium, is the active form of the protein and decarboxylated OC represents the inactive form. Gla-OC has three γ -carboxylated glutamic acid residues (Gla) at positions 17, 21, and 24 [1)] that are known to mediate strong binding to hydroxyapatite.

OC synthesis is dependent on both vitamins D and K. Vitamin D directly induces OC synthesis and vitamin K stimulates the gamma-carboxylation of glutamic acid residues. During osteoclastic resorption, binding of undercarboxylated osteocalcin (Glu-OC) to bone decreases, resulting in an increase in Glu-OC level in blood and urine.

P. D. Delmas and colleagues have shown that undercarboxylated OC is significantly increased in elderly women, suggesting an age-dependent impairment of OC γ -carboxylation. [2)] The presence of undercarboxylated OC in serum has also been associated with changes to the bone matrix and increased fragility. [3)]

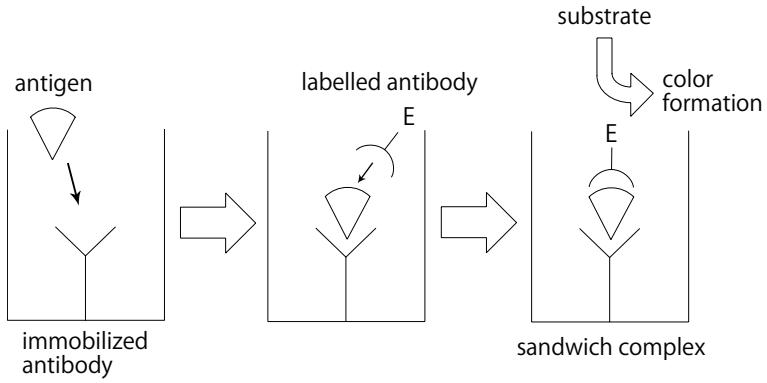
Conventional measurement of undercarboxylated OC in serum involves hydroxyapatite combination radioimmunoassay. [4)] The Undercarboxylated Osteocalcin (Glu-OC) EIA Kit uses two monoclonal antibodies that are highly specific for undercarboxylated osteocalcin (Glu-OC); one of these antibodies is attached to the assay plate and the other is peroxidase-linked. Direct measurement of Glu-OC by the Undercarboxylated Osteocalcin (Glu-OC) EIA Kit provides accurate bone metabolism data without the need for radioactivity. Furthermore, the Undercarboxylated Osteocalcin (Glu-OC) EIA Kit can be used in conjunction with the Gla-Type Osteocalcin (Gla-OC) EIA Kit (Cat. #MK111) to obtain more complete bone metabolism data.

II. Intended use

The Glu-type Osteocalcin EIA Kit is an *in vitro* enzyme immunoassay (EIA) kit intended for quantitative determination of human, bovine, rabbit, porcine, goat, sheep and monkey undercarboxylated osteocalcin (Glu-OC) in serum, plasma, urine, cell culture supernatant, and cell extracts.
This kit is for research use only; it is not for use in diagnostic or therapeutic applications.

III. Principle

The Glu-OC EIA Kit is a solid phase sandwich EIA utilizing two mouse monoclonal OC antibodies, one of which is coated onto the plate and the other of which is peroxidase (POD)-labeled. In the first step, samples and standards are added to each well and allowed to bind to the anti-Glu-OC monoclonal antibody attached to the microtiter plate. In the second step, the plate is washed and the POD-labeled monoclonal anti-OC antibody is added. During the incubation period, Glu-OC is bound by anti-Glu-OC antibody on one side and by the HRP-labeled monoclonal antibody on the other. This reaction enables the Glu-OC to be detected upon the addition of the POD substrate 3, 3', 5, 5'-tetramethylbenzidine, which results in a color development proportional to the amount of Glu-OC present in the samples and standards. The amounts of Glu-OC can then be quantitated via absorbance reading using an EIA plate reader. Accurate sample concentrations of Glu-OC can be determined by comparing their specific absorbance values with those obtained for the standards.



IV. Components

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

(2) Antibody-POD Conjugate (lyophilized) — For 11 ml
Peroxidase-Labeled Anti-OC Monoclonal Antibody

(3) Standard (lyophilized) — For 1 ml
Synthetic Glu-OC (8 ng)

(4) Sample Diluent — 11 ml x 2
BlockAce- containing PBS (with preservative)

(5) Substrate Solution (TMBZ) — 12 ml
3,3' ,5,5'-Tetramethylbenzidine solution

V. 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)

VI. Storage

4℃

VII. Precautions

- Do not mix reagents from different kit lots.
- Do not use reagents beyond the 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 with Substrate and Stop Solution. If these reagents come into contact with skin, wash thoroughly with water. Do not pipette by mouth. Do not smoke, eat, or drink in areas 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 metal. Disposable glassware or test tubes are recommended for handling the Substrate Solution. If non-disposable glassware is used, it must be acid washed and thoroughly rinsed with distilled, deionized water.
- Do not use the Substrate Solution if the color has changed to dark blue.

VIII. Protocol

1. Specimen collection and handling

Collect venous blood samples aseptically. Remove the serum from the clot or red blood cells promptly after clotting and separation. Samples containing a visible precipitant must be clarified prior to use in the assay. Do not use overly hemolyzed or lipemic specimens. Urine samples must be collected from the first excretion. Samples may be stored up to 12 hours at 4℃. If the time between sample collection and assay exceeds 12 hours, store samples at -20℃ for optimal results. Excessive freezing and thawing 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 water bath. Do not vortex or vigorously agitate.

2. Preparation of solutions

Note : The following solutions should be prepared immediately prior to use.

Solution 1. Antibody-POD Conjugate Solution
Dissolve (2) Antibody-POD Conjugate in 11 ml distilled water and mix gently and mix for 10 minutes by slowly rolling or occasionally mixing by hand, avoiding foam formation. Once reconstituted, the solution is stable for 1 week at 4℃ and for 1 month when stored at -30℃. Do not freeze and thaw.

Solution 2. Standard Solution
Dissolve (3) Standard with 1 ml distilled water. Slowly roll for approximately 10 minutes or let vials stand and occasionally mix gently. Once reconstituted, the solution is stable for 1 month when stored at -30℃. Do not repeat freeze-thaw cycles.

The Standard Solution contains 8 ng Glu-OC/ml. Prepare serial dilutions of 4, 2, 1, 0.5, 0.25, and 0.125 ng/ml by diluting the Standard Solution with (4) Sample Diluent.

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 serial dilutions of the Standard Solution 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 pipettor or similar apparatus. In order to provide highly reliable results, it is recommended that serial dilutions of the Standard Solution are placed in the 1st and 12th rows. Perform this reaction at room temperature (20 - 30℃) for 2 hours; incubation at 37℃ may compromise antigenicity. [First reaction]
2. Discard reaction solution. Wash the wells 3 times with Wash Buffer＊. Then add 100 µl of the Antibody-POD Conjugate Solution per well using an 8-channel pipettor and allow to react for 1 hour at room temperature (20 - 30℃). [Second reaction]
3. Discard reaction solution. Wash 4 times with Wash Buffer. Then add 100 µl of (5) Substrate Solution (TMBZ) per well using an 8-channel pipettor 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 for the 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 on the x-axis and absorbance on the y-axis). Use the standard curve to determine the corresponding concentrations of Glu-OC based on the sample's absorbance.

Note:

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

＊ Wash Buffer :
Wash buffer is prepared by diluting 50 ml of 10X PBS in Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021) with 450 ml of distilled water.

4. 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 Glu-OC concentration in ng/ml (horizontal axis) for the standards using an optimal fitting curve.

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 Glu-OC concentration (ng/ml) from the horizontal axis.

IX. Characteristics

1. **Range :** 0.25 - 8 ng/ml.
2. **Specificity :**
   This kit specifically measures Glu-OC with 5.0% cross-reactivity with human bone OC (likely Gla type) and 1.7% cross-reactivity with bovine bone OC (likely Gla type). This kit can be also used to measure bovine, rabbit, porcine, goat, and sheep Glu-OC, but can not measure mouse Glu-OC.
   This kit has not been tested for quantification of Glu-OC from other sources.
3. **Assay duration :**
   Three and a half hours following sample incubation.
4. **Total assay capacity :** 96 assays.
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, urine, cell culture supernatant, cell extract.
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 levels of Glu-OC approximately 2 - 3-fold.
8. **Limitation :**
   Since conditions may vary from assay to assay, a standard curve must be generated for every run. Cross-contamination between reagents will invalidate the test; use disposable pipette tips.

   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 of time between incubation steps.

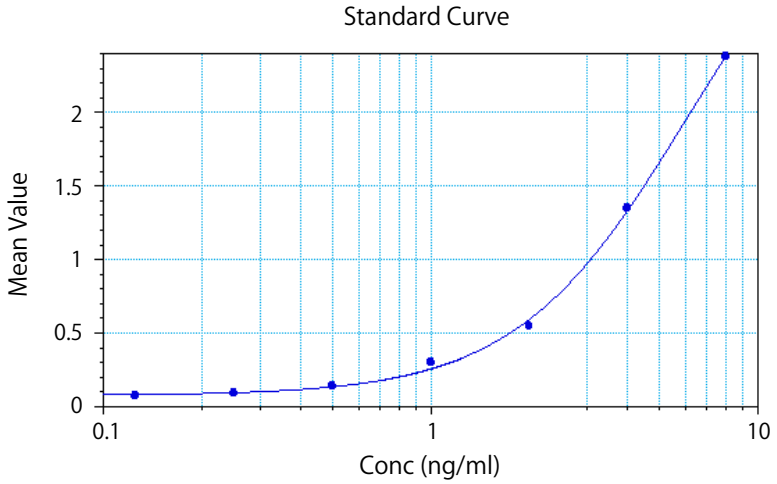
   Only samples with absorbance values falling within the range of the standard curve should be assigned a Gla-OC concentration from the curve.
9. **Notes :**
   According to the assay results using control serum, it could be possible to determine the concentration of antigen present in a biological sample. However, the measurement may be potentially disturbed by unknown organic factors in serum samples in patients with specific diseases. Similarly, a specimen obtained from an apparent healthy subject might contain unknown contaminants. 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 that the specimens be diluted with the dilution solution that is included in the kit and re-assayed.

X. Performance

1. Standard curve

The following graph shows a typical standard curve generated with this kit.
The standard curve shown below is an example only; a standard curve should be established for each individual assay.

Limit of detection: 0.25 ng/ml



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

A	B	C	D	R^2
0.0728	1.66	6.04	3.83	0.999

Std (Standards: Conc vs Mean Value)
Curve Fit Option - Fixed Weight Value

Glu-OC (ng/ml)	8.000	4.000	2.000	1.000	0.500	0.250	0.125	0.000
A_{450}	2.381	1.349	0.546	0.298	0.140	0.091	0.071	0.058

2. Reproducibility

< Intra-assay precision test (n=20) >

A reproducibility test was performed using 20 replicates of 3 samples containing different concentration of Glu-OC.

	Ave. (ng/ml)	S.D. (ng/ml)	CV (%)
Sample A	6.872	0.315	4.58
Sample B	1.796	0.079	4.40
Sample C	0.796	0.053	6.66

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

A reproducibility test was performed in three independent experiments over 3 days.

	Ave. (ng/ml)	S.D. (ng/ml)	CV (%)
Sample A	6.471	0.367	5.67
Sample B	1.585	0.150	9.46
Sample C	0.699	0.069	9.87

3. Recovery test

Equal volumes of samples in different concentrations were combined and the recovery rate was investigated using the anticipated theoretical values and the measured values

Sample A	Sample B	A+B Measured	A+B Calculated	Recovery (%)
7.90	3.42	6.03	5.66	107
7.90	1.35	4.03	4.63	87
7.90	0.63	3.03	4.27	71
3.42	1.35	2.57	2.39	108
3.42	0.63	1.95	2.03	96
1.35	0.63	1.10	0.99	111
1.35	0.38	0.77	0.87	89
0.63	0.38	0.51	0.50	101
0.38	0.23	0.23	0.31	76
0.38	0.00	0.20	0.19	104
2.91	2.62	2.44	2.77	88
2.91	0.62	1.50	1.77	85
2.62	0.36	1.33	1.49	89
2.94	2.91	3.31	2.93	113
2.94	2.62	3.03	2.78	109
2.94	0.62	2.21	1.78	124
0.61	3.73	2.07	2.17	96
0.61	0.41	0.49	0.51	95
2.90	3.73	3.17	3.32	96
2.90	0.61	1.99	1.76	113
2.90	0.41	1.99	1.66	120

(ng/ml)

4. Antibody Epitopes

Immobilized antibody : The immunogen is a synthetic human osteocalcin peptide corresponding to positions 14 to 30. The epitope is likely glutamic acid at positions 21 and 24.

Labeled antibody : The epitope corresponds to amino acid 21 - 31 of bovine osteocalcin.

Intact Osteocalcin	1	17	21	24		49
Fragmented Osteocalcin	1	17	21	24	43	
			21	24		49
			21	24	43	

The above forms that have no Gla-residues will be detected with this ELISA kit.

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

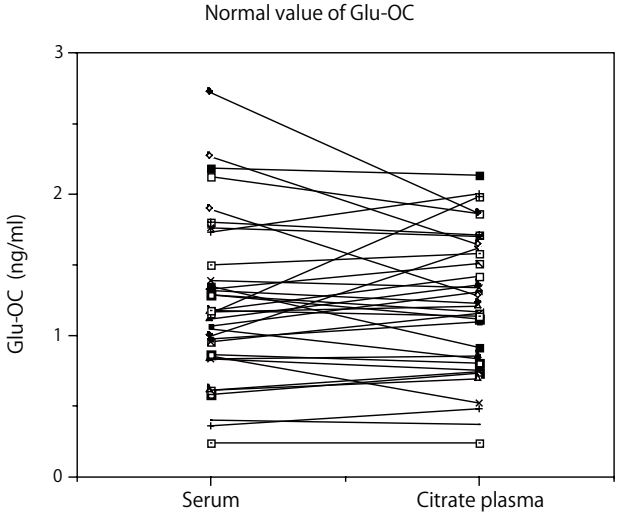
Treatment with hydroxyapatite (HAP) enables absorption of activated (carboxylated) OC but not decarboxylated OC. Active OC can be recovered through elution by using a phosphate buffered solution. This ELISA kit can detect decarboxylated OC.

Sample No.	Serum Glu-OC	HAP treated serum Glu-OC	Glu-OC eluted with phosphate buffer
1	0.645	0.755	0.000
2	1.312	1.431	0.000
3	0.920	1.228	0.000
4	0.315	0.572	0.000
5	0.196	0.583	0.000
6	0.159	0.189	0.000
7	0.933	2.739	0.000
8	0.850	1.158	0.000
9	0.319	0.489	0.000
10	0.777	1.856	0.000
11	0.143	0.172	0.000

(ng/ml)

6. Comparison Glu-OC value between citrated plasma and serum

No significant difference between the Glu-OC content in serum and citrated plasma is observed. Normal values should be determined with a statistically adequate number of samples considering age.



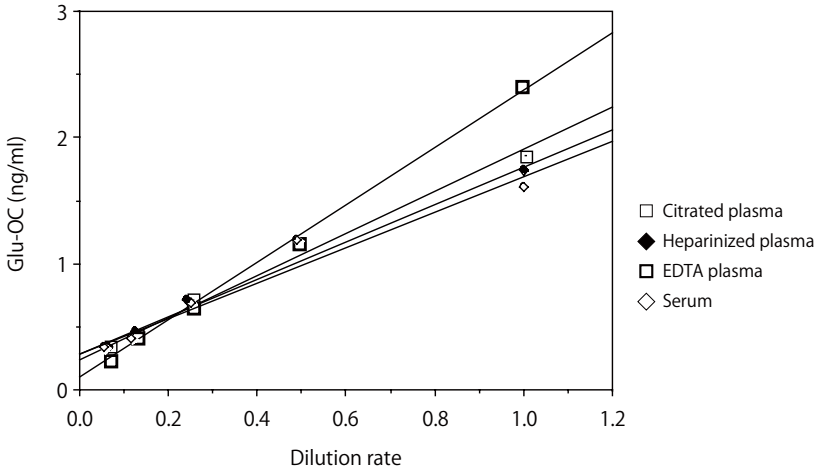
Average	1.236 ng/ml	1.210 ng/ml
S.D.	0.569	0.492

(n=35, age average 29)

7. Effect of anticoagulants

The effect of several anticoagulants was evaluated by comparing the dilution curve of normal human samples treated with different anticoagulants.

< Sample dilution curve and effect of anticoagulants >

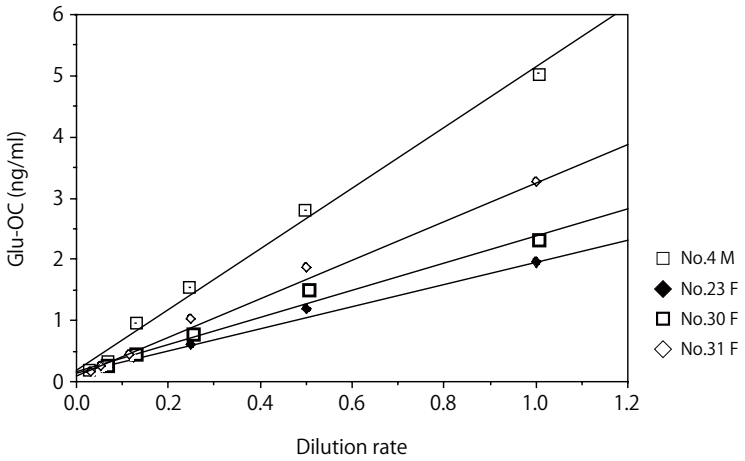


y = 1.6687x + 0.23954	r^2 = 0.992	Citrated plasma
y = 1.4809x - 0.28583	r^2 = 0.979	Heparinized plasma
y = 2.2825x +9.8195e-2	r^2 = 0.995	EDTA plasma
y = 1.4081x + 0.28226	r^2 = 0.964	Serum

8. Comparison of different serum samples

The dilution curves from four different serum samples were compared.

< Glu-OC serum dilution comparison >

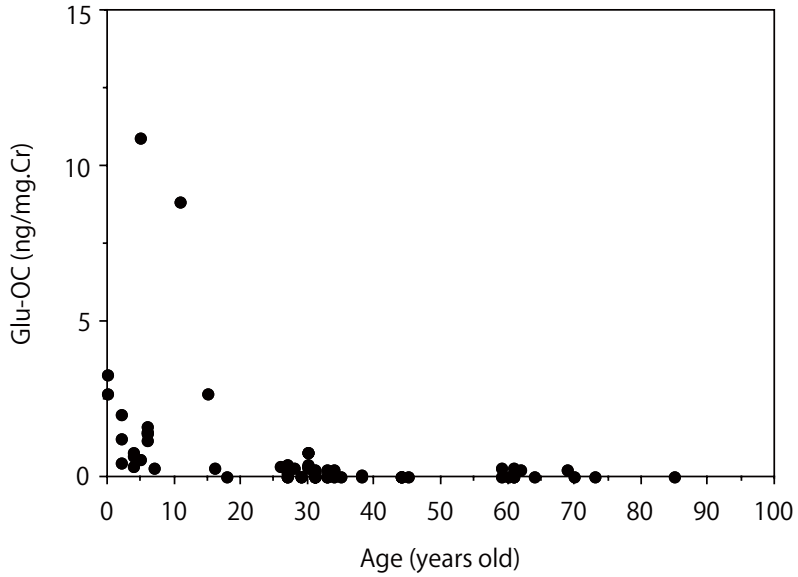


y = 4.9676x + 0.18873	r^2 = 0.996	No.4 M
y = 1.8127x + 0.14717	r^2 = 0.988	No.23 F
y = 2.2100x + 0.17442	r^2 = 0.976	No.30 F
y = 3.1629x + 9.4473e - 2	r^2 = 0.996	No.31 F

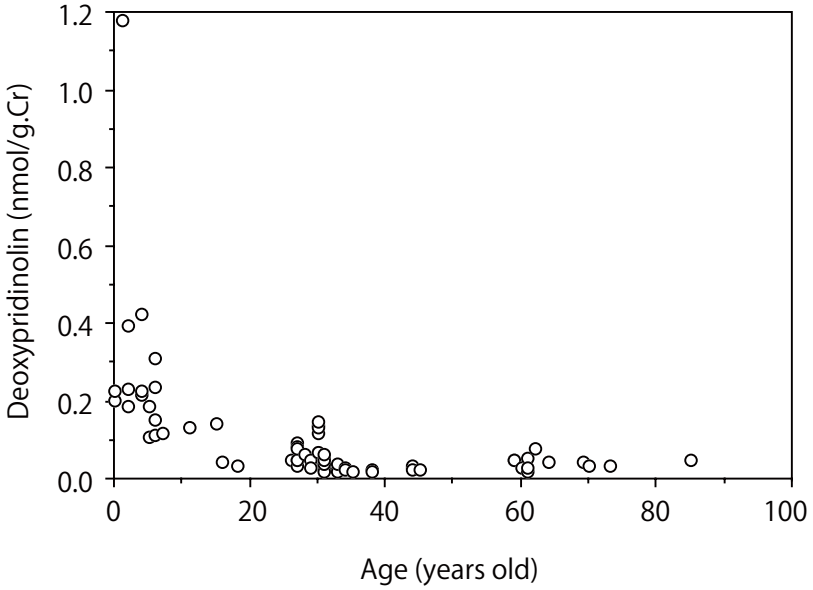
9. Change in urine Glu-OC value and deoxypyridinoline in aging

Human urine samples from subjects varying in age were collected early in the morning.
Glu-OC value and deoxypyridinoline (PYRILINKS-D Assay) in urine were normalized to creatinine level (Cr). Both values decrease as a function of age.

< Urinary Glu-OC/Cr levels in normal subjects >



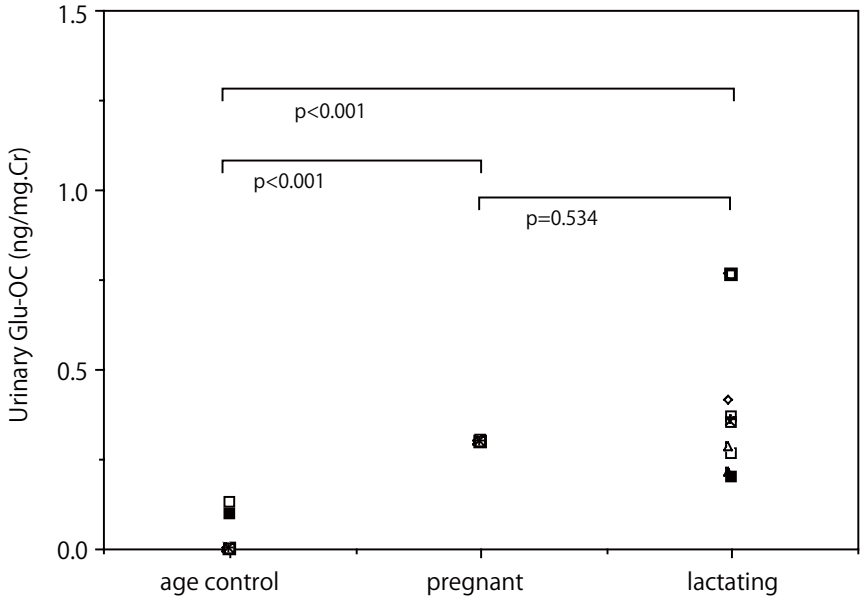
< Urinary deoxypridinolin/Cr in normal subjects >



10. Urinary Glu-OC values in lactating and pregnant women

Urine samples were collected from 25 - 35 year-old females early in the morning. Urinary Glu-OC value in lactating and pregnant women is significantly higher than that in age-matched controls.

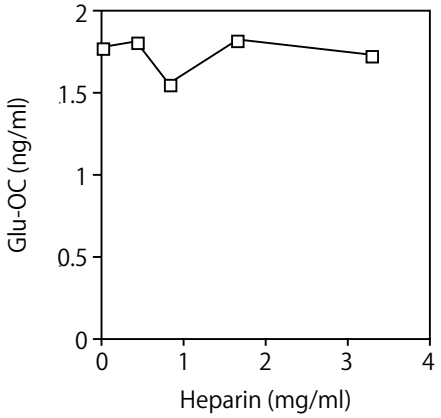
Urinary Glu-OC values in control, pregnant, and lactating women

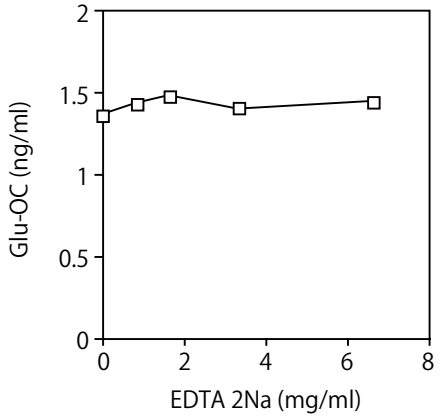


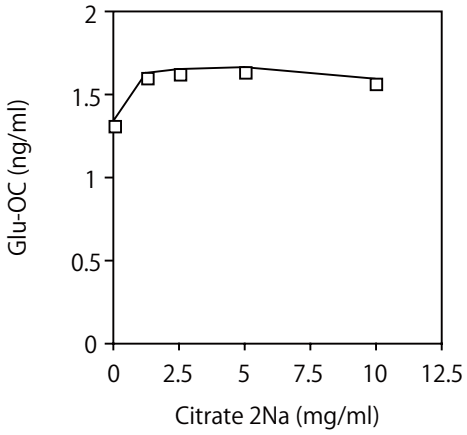
Average	0.028	0.302	0.399
S.D.	0.053	0.007	0.413
n=	8	2	10

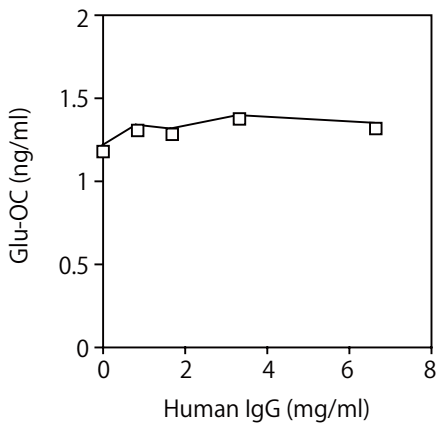
11. Influence of coexisting substances

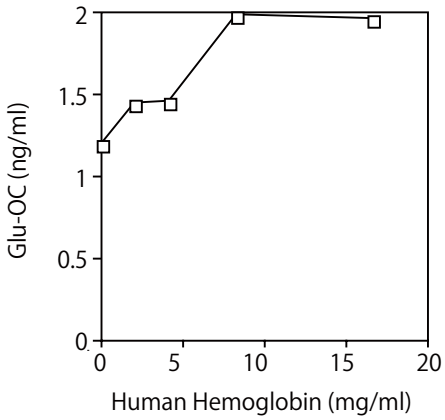
The ratio of sample to coexisting substance is 4 : 1 (by volume). The final concentration of coexisting substance is shown on the x-axis.

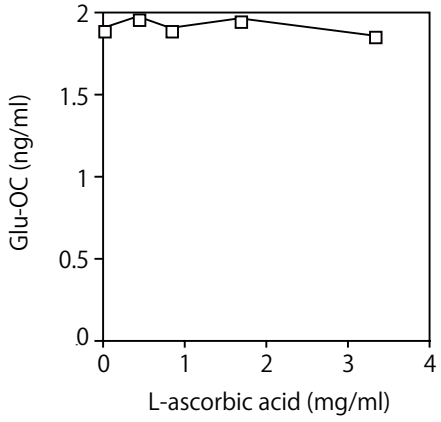


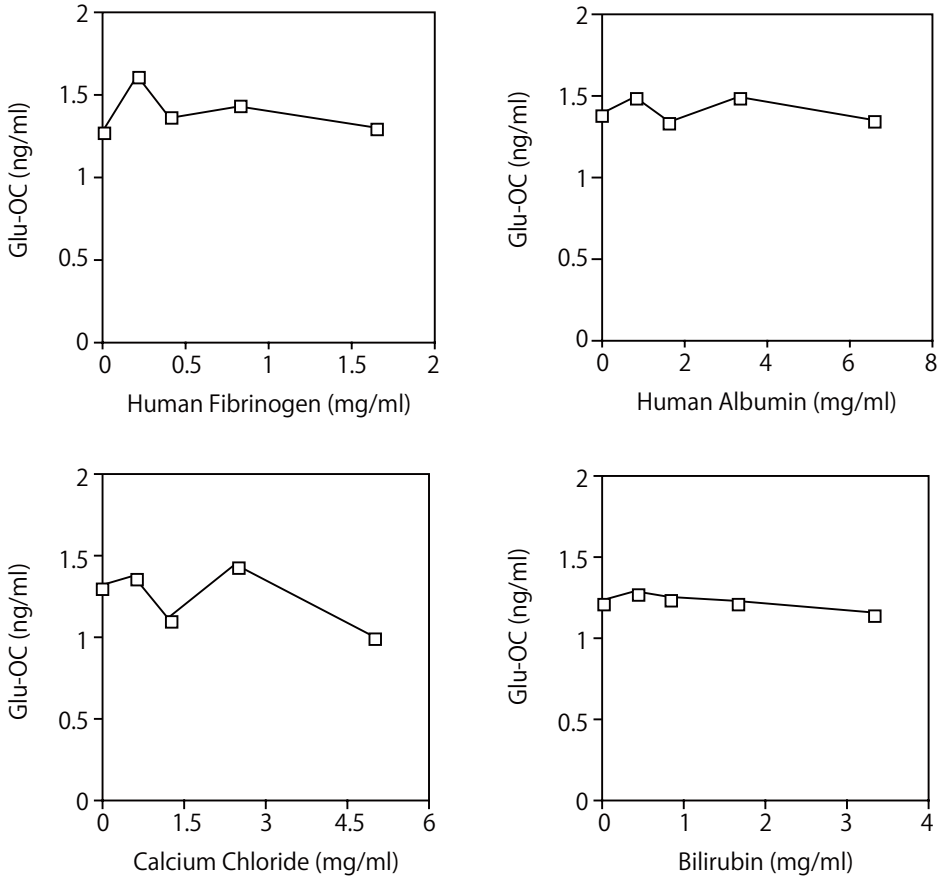






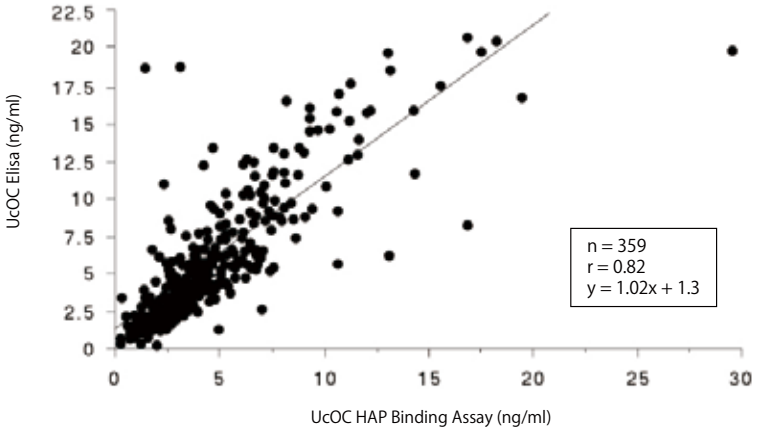






12. Comparison with hydroxyapatite combination RIA method

Glu-OC concentration data derived from the Undercarboxylated Osteocalcin (Glu-OC) EIA Kit correlate well with data derived from the hydroxyapatite combination RIA method.[3, 4, 6)]



XI. Related Products

Gla-Type Osteocalcin (Gla-OC) EIA Kit (Cat. #MK111)
Mouse Glu-Osteocalcin High Sensitive EIA Kit (Cat. #MK129)
Human Gla-Osteocalcin High Sensitive EIA Kit (Cat. #MK128)
Rat Glu-Osteocalcin High Sensitive EIA Kit (Cat. #MK146)
Pig Glu-Osteocalcin EIA Kit (Cat. #MK149)
Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021)

XII. References

1) Poser J W, *et al*. *J Biol Chem*. (1980) **255**: 8685.
2) Plantalech L, *et al*. *J Bone Miner Res*. (1991) **6**: 1211.
3) Szulc P, *et al*. *J Clin Invest*. (1993) **91**: 1769.
4) Price P A, *et al*. *J Biol Chem*. (1980) **255**: 2938.
5) Koyama N, *et al*. *J Immunol Meth*. (1991) **139**: 17.
6) Vernaud P, *et al*. *J Clin Endocrinol Metab*. (1997) **82**: 719-724.

XIII. Protocol Summary

1. Prepare all reagents as directed.
2. Bring all reagents to room temperature and prepare solutions.
3. Add 100 μl of Standard or sample to appropriate wells, and incubate for 2 hours at room temperature (20 - 30℃).
4. Remove sample solution and wash the wells 3 times with 400 μl of PBS.
5. Add 100 μl of Antibody-POD Conjugate Solution into wells and incubate at room temperature (20 - 30℃) for 1 hour.
6. Aspirate solution from wells. Wash 4 times with 400 μl of PBS per well, aspirating thoroughly in between washes.
7. Add 100 μl of Substrate Solution (TMBZ) to each well. Incubate 10 - 15 minutes at room temperature (20 - 30℃).
8. Add 100 μl of Stop Solution to all wells. Mix gently.
9. Read absorbance at 450 nm as soon as possible.