Undercarboxylated Osteocalcin (Glu-OC) EIA KIT

An enzyme immunoassay kit for the quantitative determination of Undercarboxylated Osteocalcin (Glu-OC)

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

Code No. MK118
For 96 assays

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Introduction
Osteocalcin (OC), also known as bone g-carboxylglutamic acid protein, is a vitamin K-dependent Ca\(^{2+}\)-binding protein of molecular weight 5900. 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. The synthesis of OC depends on both the vitamin D and vitamin K. Vitamin D induces directly the OC synthesis, and vitamin K stimulates the \(\gamma\)-carboxylation of glutamic acid residues. For resulting these enzymes deficiency or osteoclastic bone resorption, undercarboxylated osteocalcin (Glu-OC) decreases binding to the bone and increases circulating in blood stream and urine.

P.D. Delmas and his co-worker have previously shown that undercarboxylated OC which does not bind to hydroxyapatite, is significantly increased in elderly women, suggesting an age dependent impairment of the \(\gamma\)-carboxylation of OC (2). Further they suggest that serum undercarboxylated OC reflects some change in bone matrix associated with increased fragility (3). Until now, measurement of undercarboxylated OC in serum sample is usually performed by hydroxyapatite combination radio immunoassay (4).
Undercarboxylated Osteocalcin (Glu-OC) EIA Kit Manual

We raised the monoclonal antibody specific to undercarboxylated osteocalcin and constructed ELISA system. This undercarboxylated osteocalcin EIA Kit utilizes a novel set of monoclonal antibodies highly reactive to the decarboxylated osteocalcin (Glu-OC) and less reactive to carboxylated at positions 17,21,24 form. Direct measurement of Glu-OC by this EIA system provides as useful leads of clinical information of bone metabolism as Gla-type osteocalcin EIA(5) (Takara Cat.#MK111).

Intended use
The Glu-type Osteocalcin EIA Kit is an in vitro enzyme immunoassay (EIA) kit for quantitative determination of human, bovine, rabbit, porcine, goat, sheep undercarboxylated osteocalcin(Glu-OC) in serum, urine, and other biological fluids. This kit is for research use only. It is not for use in diagnostic or therapeutic procedures.

Principle
The Glu-OC EIA Kit is a solid phase EIA based on a sandwich method that utilizes two mouse monoclonal anti undercarboxylated OC antibodies to detect Glu-OC by two-step procedure. One of the mouse monoclonal anti-undercarboxylated OC is immobilized onto the microtiter plate and blocked against non-specific binding. Samples and standards are added each wells and incubated. The second step is to wash the plate and to add the second anti-OC labelled with peroxidase (POD). During this incubation, Glu-OC is bound to anti-undercarboxylated OC (solid phase) on one side and tagged on the other by POD-anti OC. The reaction between POD and substrate (H₂O₂ and 3,3′,5,5′ tetramethylbenzidine) results in colour development with intensifies proportional to the amount of Gla-OC present in samples and standards. The amount of Glu-OC can be quantitated by measuring the absorbance using an EIA plate reader. Accurate sample concentrations of Glu-OC can be determined by comparing their specific absorbances with those obtained for the standards plotted on a standard curve.
Reagents and materials

Each Glu-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 (8 well x 12 strips)
The plate coated with murine monoclonal antibody to Glu-OC.
Store at 2 - 8°C.

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

Vial 3. Standard - 1 vial (8 ng x 1)
The vial contains lyophilized synthetic Glu-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 Na₂HPO₄·12H₂O and 0.2 grams of KH₂PO₄ in 1000 ml of distilled water.)
   - Stop Solution : 1 N H₂SO₄

2. Materials
   - Precision pipettes with disposable tips: 20 and 100 μl micropipettes, 10 - 200 μl adjustable multiwell pipetter or 20 and 100 μl multiwell pipettes
   - 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 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 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 glassware 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.

Specimen collection and handling
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. Urine samples must be collected in the first excretion. Samples may be stored up to 24 hours at 4°C. If the length of time between sample collection and assay is to exceed 24 hours, samples should be stored frozen under -20°C 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 directly before use.

Solution 1. Antibody-POD Conjugate Solution
Dissolve the contents of Vial 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 (Vial 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 8 ng Glu-OC/ml.

Stability of solutions
Solution 1. The reconstituted lyophilisate is stable for 1 week at 4°C and for 1 month when stored at -30°C.
Solution 2. The reconstituted lyophilisate is stable for 1 month when stored at -30°C.
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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]
- **Sample incubation:** Pipette 100 μl sample and standard (Solution 2) into one well within 5 minutes. Mix, seal the microtiter plate (e.g. with a foil) and incubate for 2 hours at room temperature (20 - 25°C). Do not incubate at 37°C with serum because antigen denaturalization (proteolysis) may occur.
- 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.
- **Antibody-POD conjugate incubation:** Pipette 100 μl of Antibody-POD Conjugate Solution (Solution 1) into one well, mix, seal the microtiter plate (e.g. with a 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 minutes.
- Add 100 μl Stop Solution (1N H₂SO₄) into each well in the same order as for 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 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.
  
  **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 Glu-OC concentration in ng/ml (horizontal axis) for the standards using 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.
Performance characteristics

1. Range of standard curve: 0.25-8 ng/ml.

2. Specificity: This kit specifically measures Glu-OC with 5.0% crossreactivity with human bone OC (probably Gla type) and 1.7% crossreactivity with bovine bone OC (probably Gla type). This kit can be also used to measure bovine, rabbit, porcine, goat and sheep Glu-OC, but not to measure mouse Glu-OC. The application of this kit for quantitating Glu-OC from other sources has not been tested.

3. Assay duration: Three and a half hour after 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,

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 Glu-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.
Basal data

1. Typical standard curve

Typical Standard Curve
(Do Not Use To Calculate Unknowns)

Curve Fit: Linear  Corr. Coeff: 0.998

\[ y = A + B \times x \]
\[ A = 0.00791 \quad B = 0.230 \]

2. Intra-assay precision (n=20)
Assay was carried out with 20 replicates of 3 samples containing different concentration of Glu-OC.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ave. (ng/ml)</th>
<th>S.D. (ng/ml)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A</td>
<td>6.872</td>
<td>0.315</td>
<td>4.58</td>
</tr>
<tr>
<td>Sample B</td>
<td>1.796</td>
<td>0.079</td>
<td>4.40</td>
</tr>
<tr>
<td>Sample C</td>
<td>0.796</td>
<td>0.053</td>
<td>6.66</td>
</tr>
</tbody>
</table>

Inter-assay precision (performance 3 times)
Assay to assay precision with one laboratory was evaluated in three independent experiments over 3 days.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ave. (ng/ml)</th>
<th>S.D. (ng/ml)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A</td>
<td>6.471</td>
<td>0.367</td>
<td>5.67</td>
</tr>
<tr>
<td>Sample B</td>
<td>1.585</td>
<td>0.150</td>
<td>9.46</td>
</tr>
<tr>
<td>Sample C</td>
<td>0.699</td>
<td>0.069</td>
<td>9.87</td>
</tr>
</tbody>
</table>
3. Recovery test
The recovery of Glu-OC was tested by adding two samples out of ten different levels in various matrices.

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

4. Epitope of the antibodies of this kit
The first antibody: Immunogen is human osteocalcin synthetic peptide 14 position to 30 position. Epitope is probably 21 and 24 position glutamic acid residue.

Labeled antibody: Bovine osteocalcin, 21-31 amino acid residue.

<table>
<thead>
<tr>
<th>Intact Osteocalcin</th>
<th>1</th>
<th>17</th>
<th>21</th>
<th>24</th>
<th>49</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment Osteocalcin</td>
<td>1</td>
<td>17</td>
<td>21</td>
<td>24</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>24</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>24</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>

The above forms having no Gla-residue will be detected with this ELISA kit.
5. Effect of hydroxyapatite powder treatment on serum Glu-OC value
By treatment with hydroxyapatite, OC that binds to bone (active form) will be absorbed with hydroxyapatite, but decarboxylated OC will not be absorbed. Active form of OC was recovered from hydroxyapatite by eluting with phosphate buffer solution. This ELISA system is useful for direct detection of decarboxylated OC.

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

6. Correlation of citrate plasma and serum value of Glu-OC
There is no significant difference between serum and citrate plasma Glu-OC level serum. Normal value should be determined with statistically adequate number of samples considering age.

![Normal value of Glu-OC](image)

Average Glu-OC conc. (ng/ml)
- Serum: 1.236 ng/ml
- Citrate plasma: 1.210 ng/ml

S.D.
- Serum: 0.569
- Citrate plasma: 0.492

(n=35, age average 29)
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)

- **Sample dilution curve and effect of anticoagulants**

![Graph showing dilution curves for different anticoagulants]

\[
y = 1.6687x + 0.23954 \quad r^2 = 0.992 \quad \text{Citrate plasma}
\]
\[
y = 1.4809x - 0.28583 \quad r^2 = 0.979 \quad \text{Heparinized plasma}
\]
\[
y = 2.2825x + 9.8195e-2 \quad r^2 = 0.995 \quad \text{EDTA plasma}
\]
\[
y = 1.4081x + 0.28226 \quad r^2 = 0.964 \quad \text{Serum}
\]

8. Parallelism of four different Glu-OC EIA serum samples

The dilution curves of four different serum samples were compared.

- **Glu-OC EIA serum sample dilution parallelism**

![Graph showing dilution curves for different serum samples]

\[
y = 4.9676x + 0.18873 \quad r^2 = 0.996 \quad \text{No.4 M}
\]
\[
y = 1.8127x + 0.14717 \quad r^2 = 0.988 \quad \text{No.23 F}
\]
\[
y = 2.2100x + 0.17442 \quad r^2 = 0.976 \quad \text{No.30 F}
\]
\[
y = 3.1629x + 9.4473e-2 \quad r^2 = 0.996 \quad \text{No.31 F}
\]
9. Alteration of urine Glu-OC value and deoxypyridinoline in aging

Human urine samples from various age levels were collected early in the morning. Glu-OC value and deoxypyridinoline (PYRILINKS-D Assay) in urine were corrected by creatinine. Following aging, these indicators decrease remarkably.

- Urinary Glu-OC/Cr in normal person

![Graph showing the decrease of Urinary Glu-OC/Cr with age]

- Urinary deoxypyridinolin/Cr in normal person

![Graph showing the decrease of Urinary deoxypyridinolin/Cr with age]
10. Comparison of urinary Glu-OC value in lactating and pregnancy with normal
Urine samples were collected from 25-35 year-old female early in the morning. Urinary Glu-OC value in lactating and pregnancy is significantly higher than that in control female corresponding to age respectively.

<table>
<thead>
<tr>
<th></th>
<th>Average</th>
<th>S.D.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>age control</td>
<td>0.028</td>
<td>0.053</td>
<td>8</td>
</tr>
<tr>
<td>pregnant</td>
<td>0.302</td>
<td>0.007</td>
<td>2</td>
</tr>
<tr>
<td>lactate</td>
<td>0.399</td>
<td>0.413</td>
<td>10</td>
</tr>
</tbody>
</table>

Urine Glu-OC (ng/mg.Cr)
11. Influence of coexistence

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

- Citrate 2Na (mg/ml)
- Heparin (mg/ml)
- EDTA 2Na (mg/ml)
- Human Hemoglobin (mg/ml)
- Human IgG (mg/ml)
- L-ascorbic acid (mg/ml)
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12. Correlation with hydroxyapatite combination RIA method

It is confirmed that serum Glu-OC assay with this EIA system has good correlation with that with RIA method by Dr. Delmas (3)(4)(6).

UcOC Elisa (ng/ml)

UcOC HAP Binding Assay (ng/ml)

n = 359
r = 0.82
y = 1.02x + 1.3
Storage and Stability
This kit is shipped at 2-8°C and should be stored at 2-8°C if not used. Under this condition, the kit is stable until the expiry date printed in the box label.

References

Protocol summary
1. Prepare all reagents as directed in the Package Insert.
2. Bring all reagents to room temperature and prepare the solutions.
3. Add 100 μl of Standard or sample to appropriate wells, and incubate for 2 hours at room temperature (20 - 25°C).
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 for 1 hour.
6. Aspirate solution from wells. Wash 4 times with 400 μl of PBS per wells, aspirating thoroughly between washes.
7. Add 100 μl of OPD Substrate Solution to each well. Incubate 15 minutes at room temperature.
8. Add 100 μl of Stop Solution to all wells. Mix gently.
9. Read at 450 nm as soon as possible.