Cat. # MK146

For Research Use

TaKaRa

Rat Glu-Osteocalcin High Sensitive EIA Kit

Product Manual

v201607Da

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I. Description

Osteocalcin, which contains two or three γ-carboxyglutamate (Gla) residues and has a molecular weight of approximately 5,900 Daltons, is known as a vitamin K-dependent calcium-binding non-collagen protein. Specifically produced by only osteoblasts, osteocalcin has been used as one of the osteoblast markers. The rat osteocalcin consists of a total of 50 amino acids. Human, bovine, rabbit, and other species have a osteocalcin with 49 amino acids. The three glutamate residues at positions 17, 21, and 24 of the amino acid chain are carboxylated, forming a calcium pocket that allows osteocalcin to bind to bone matrix.

Osteoblasts generally produce osteocalcin with all three of its glutamate residues carboxylated (Gla-OC), affording the protein its ability to bind with bone matrix. During bone metabolism, osteocalcin is released from bone matrix through the actions of various enzymes, including one produced by osteoclasts. Most of the three glutamate residues are decarboxylated on osteocalcin (Glu-OC) when it is released into blood from bone. Therefore, osteocalcin is present in blood in both Gla and Glu forms and is made up of a wide range of molecular species, from full-length to fragmented molecules.

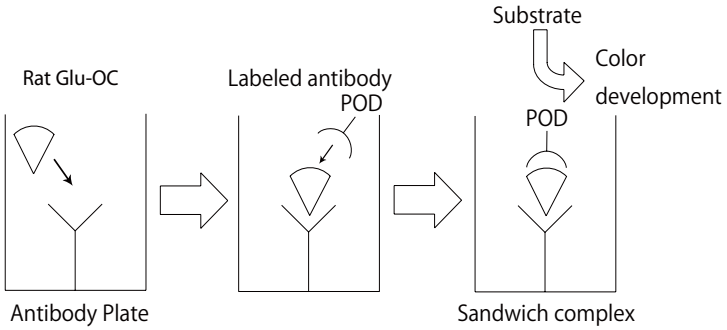
		10	20	30	40	50
Human	1	YLYQWLGAPV	PYPDPLEPRR	EVCELNPDCD	ELADHIGFQE	AYRRFYGP-V
Bovine	1	YLDHWLGAPA	PYPDPLEPKR	EVCELNPDCD	ELADHIGFQE	AYRRFYGP-V
Rat	1	YLNNGLGAPA	PYPDPLEPHR	EVCELNPNCD	ELADHIGFQD	AYKRIYGTTV
Mouse	1	YL----GASV	PSPDPLEPTR	EQCELNPACD	ELSDQYGLKT	AYKRIYGITI
Chicken	1	YAQDSGVAGA	P-PNPLEAQR	EVCELSPDCD	ELADQIGFQE	AYRRFYGP-V
Monkey	1	YLYQWLGAPA	PYPDPLEPKR	EVCELNPDCD	ELADHIGFQE	AYRRFYGP-V
Pig	1	YLDHGLGAPA	PYPDPLEPRR	EVCELNPDCD	ELADHIGFQE	AYRRFYGI-A

Figure 1. The Amino Acid Sequence (Primary Structure) of Osteocalcin in Animal Species

As can be seen in Figure 1, rat osteocalcin is most homologous to human osteocalcin,and is frequently used in bone-related experiments due to rats' short life, ease of handling and the ability to guarantee the number of individuals in the experimental system.[1, 2)]There are several scholarly articles in which osteocalcin has been measured as a marker of bone formation. Originally, RIA using iodine-labeled osteocalcin were used, but in recent years, EIA using enzymes have also been constructed.[3)] However, there are almost no examples in which undercarboxylated deactivated (Glu-type) osteocalcin has been measured separately from Gla-type osteocalcin. The ability to carry out differential determination could enable osteocalcin to become two contradictory markers, for both bone formation and bone resorption.

Rat Glu-Osteocalcin High Sensitive EIA Kit is a sandwich-type EIA kit in which rat osteocalcin C terminal region recognition specific antibody is the capture antibody on a solid plate and a monoclonal antibody that is specific to the Glu residues that straddle positions 21 and 24 of osteocalcin is arranged as the detection antibody. This product makes high-sensitivity measurement of minor antigens and maintenance of stable reproducibility possible. The use of a 96-well plate makes it possible to assay many sample treatments. Moreover, as it has the same capture antibody as the Rat Gla-Osteocalcin High Sensitive EIA Kit (Cat. #MK126), the kits may be used together for simultaneous Gla/Glu detection, thereby making simultaneous monitoring of bone formation and resorption possible.

II. Principle



III. Components

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

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 Glu-type osteocalcin (Rat Glu-OC) in rat biological samples.

VII. Protocol

1. Sample

- Suitable samples include rat serum, ascites, cell culture supernatant, cell extracts, 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 rat serum samples is 20 - 100 fold.
- Values for hemolyzed serum tend to be markedly lower.

2. Preparation of Solutions

- Antibody Coated Microtiter Plate
  Allow the (1) Anti-Rat 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.
- Rat Glu-OC Standard Solution
  Add 1 ml of distilled water to the lyophilized (3) Standard to reconstitute it (8.0 ng/ml). Dilute the Standard with (4) Sample Diluent before use to prepare fresh serial dilutions of Standard Solution at concentrations of 8.0, 4.0, 2.0, 1.0, 0.5, 0.25, and 0.125 ng/ml. Use Sample Diluent as the 0-concentration standard.
  The dissolved Rat Glu-OC standard solution (8 ng/ml) is stable for up to 1 week after preparation when stored at 4℃, or for up to 1 month at -20℃.
- 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 min) 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 min. [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 Rat Glu-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 (Rat Glu-Osteocalcin High Sensitive EIA Kit)

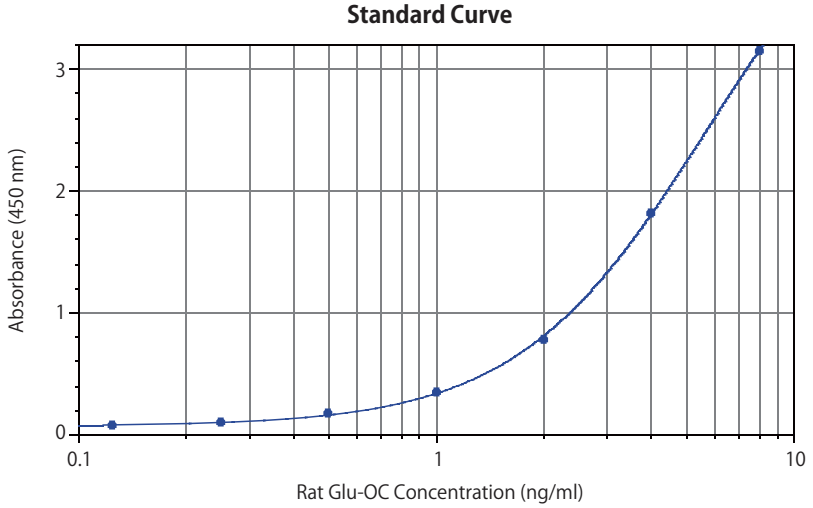
The following shows a typical standard curve of this kit as an example.
The standard curve for calculation needs to be established in each assay.

Minimum Sensitivity of Detection: 0.125 ng/ml

Curve Fit：4-Parameter　　Corr. Coeff：1.00

$y = (A - D)/\{1 + (x/C)^B\} + D$

A = 0.0656　　B = 1.63　　C = 5.72　　D = 4.94



Concentration of Rat Glu-OC (ng/ml)	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.0
A_{450}	3.150	1.819	0.782	0.350	0.170	0.094	0.071	0.053

(Color Development Time: 15 min)

2. Reproducibility

<Intra-assay precision test (n=8)>

A reproducibility test was performed with 8 replicates, using 3 different concentrations of rat serum.

Sample	Mean Value (ng/ml)	CV (%)
Control A	4.418	4.8
Control B	1.976	2.6
Control C	0.811	4.1

<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 Value (ng/ml)	CV (%)
Control A	4.096	6.3
Control B	1.867	4.1
Control C	0.794	2.4

3. Recovery test

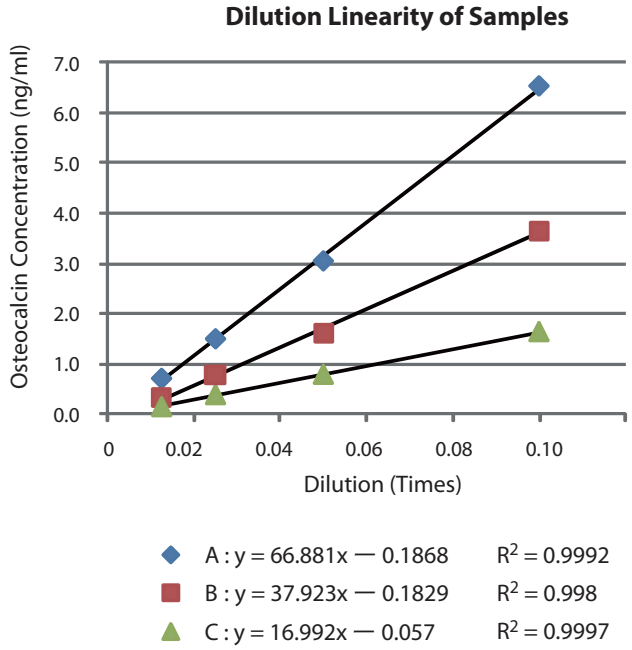
Equal volumes of samples in different concentrations were combined and assayed. The result of each mixture was compared with the theoretical value to determine the recovery rate.

Sample A	Sample B	A + B (Theoretical Value)	A + B (Actual Value)	Recovery Rate (%)
9.153	3.698	6.426	6.721	104.6
9.153	1.742	5.448	5.505	101.1
9.153	0.880	5.017	5.087	101.4
9.153	0.576	4.865	4.984	102.5
9.153	0.315	4.734	4.676	98.8
4.167	3.698	3.933	4.381	111.4
4.167	1.742	2.955	3.172	107.4
4.167	0.880	2.524	2.852	113.0
4.167	0.576	2.372	2.630	110.9
4.167	0.315	2.241	2.483	110.8
2.098	3.698	2.898	3.208	110.7
2.098	1.742	1.920	2.119	110.4
2.098	0.880	1.489	1.938	130.2
2.098	0.576	1.337	1.432	107.1
2.098	0.315	1.207	1.425	118.1
1.058	3.698	2.378	2.496	105.0
1.058	1.742	1.400	1.523	108.8
1.058	0.880	0.969	1.094	112.9
1.058	0.576	0.817	0.844	103.3
1.058	0.315	0.687	0.775	112.9
0.518	3.698	2.108	2.194	104.1
0.518	1.742	1.130	1.231	108.9
0.518	0.880	0.699	0.817	116.9
0.518	0.576	0.547	0.518	94.7
0.518	0.315	0.417	0.475	114.0

Average Recovery Rate: 108.8%
Units: ng/ml

4. Linearity of Rat Serum

10, 20, 40, and 80 times dilutions from 3 different normal rat serum (A, B, and C) were assayed using this kit and dilution linearity was confirmed.



5. Effects of Sample Hemolysis

Blood collected from individual 4 week old rats was divided in half. The first half was promptly centrifuged and then serum was collected. The second half was passaged through an injection needle a few times to induce hemolysis and then centrigued to collect the hemolysed serum. All samples were assayed simultaneously. Serially-diluted solutions were prepared (10, 20, 40, and 80 times) and assayed in order to determine the optimal dilution ratio. Additionally, measurement with a Rat Gla-Osteocalcin High Sensitive EIA Kit (Cat. #MK126) was carried out simultaneously in order to investigate whether hemolysis had any effect on the two kits.

The results for the 20 times diluted serum are shown for Cat. #MK146 and the results for the 40 times diluted serum are shown for Cat. #MK126.

Kit	MK146 Rat Glu-OC		MK126 Rat Gla-OC	
Dilution	20 times Dilution		40 times Dilution	
Sample	Normal Serum	Hemolytic Serum	Normal Serum	Hemolytic Serum
	Serum Concentration Converted Value (ng/ml)			
Rat 1	56.1	15.8	448.9	158.5
Rat 2	38.4	3.9	247.4	45.0
Rat 3	40.5	Below Sensitivity	331.7	17.0
Rat 4	79.5	Below Sensitivity	549.0	9.8

Results:
A tendency for hemolysis to cause both Gla-type and Glu-type osteocalcin measurements to be extremely low was observed in all individuals.
It is recommended that hemolytic serum be excluded from measurement or that the effects of hemolysis on the assay be taken into consideration.

6. Cross-Reactivity with Serum of Various Animal Species

Cross-reactivity with the serum of various animals was examined.

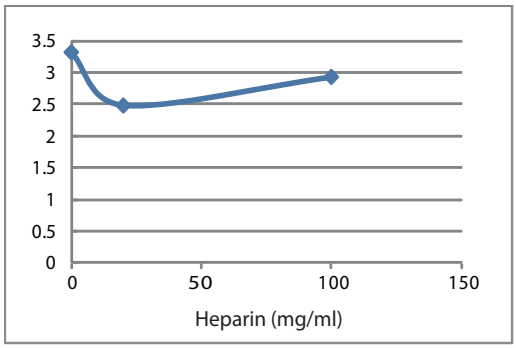
		Dilution			
Sample		10X	50X	250X	1,250X
Rat	Male, 9 Weeks	4.000	0.818	0.141	0.067
	Retired, Female, Day 3 after Giving Birth	0.434	0.093	0.058	0.057
	Male, 12 Weeks	2.713	0.394	0.095	0.061
		1.128	0.196	0.073	0.055
	Male, 24 Days	4.000	1.589	0.244	0.080
Fetal Calf (Before Inactivation)		0.054	0.050	0.051	0.054
Cattle	Less than 3 Years	0.055	0.052	0.052	0.051
Human		0.061	0.054	0.051	0.048
Horse		0.116	0.073	0.060	0.057
Dog		0.060	0.056	0.055	0.052
Pig	Male, 8 months	0.066	0.058	0.056	0.056
Sheep		0.094	0.060	0.056	0.053
Goat		0.068	0.058	0.053	0.055
Rabbit	Male, 12 weeks	0.082	0.064	0.056	0.055
Rabbit	Male, 12 weeks	0.078	0.065	0.056	0.055
Guinea Pig	Male, 12 weeks	0.068	0.056	0.053	0.052

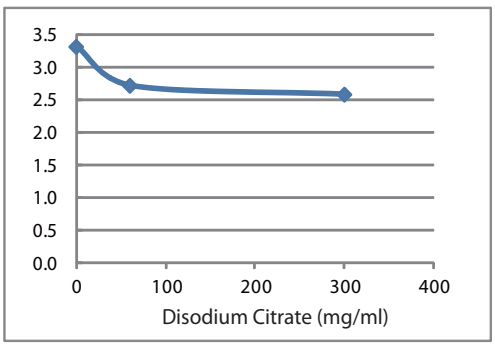
Absorbance at A450nm

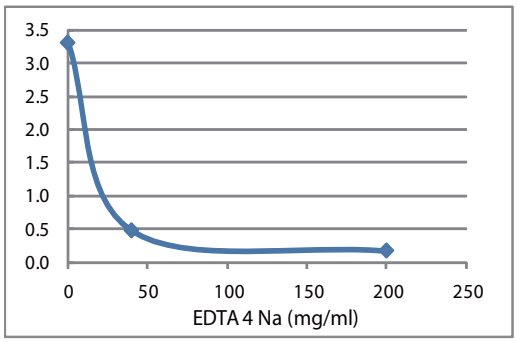
Results:
The measurements with this kit are rat-specific, and no cross-reactivity with other animals (human included) was observed. A tendency towards high concentrations with the blood of young rats was observed.

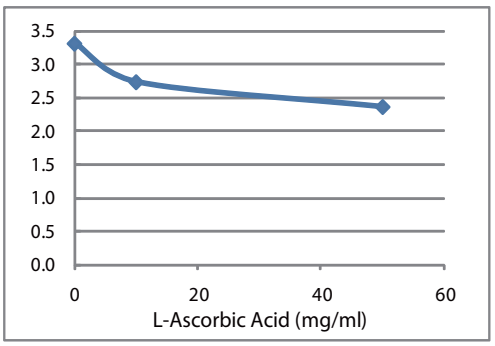
7. Effects of Coexisting Substances

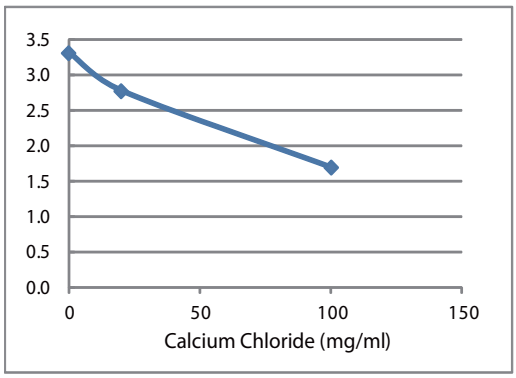
1 part by volume of the substance being tested was added to 9 parts by volume of osteocalcin Standard Solution, and the effects on the reaction were examined. The final concentration of the test substance is shown on the horizontal axis of the graph. The concentration of Glu-osteocalcin measured is shown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











Results:
EDTA 4 Na and calcium chloride tend to interfere with the reaction.

8. Comparison of Rat Glu-OC Competitive EIA Kit (Cat. #MK122)＊ and Rat Glu-Osteocalcin High Sensitive EIA Kit (Cat. #MK146)

A comparison of the Rat Glu-OC Competitive EIA Kit (Cat. #MK122)＊ and this product (Cat. #MK146) was carried out with three rat serum samples.

＊：Cat. #MK122 is already final sales.

	MK146 (ng/ml)	MK122 (ng/ml)
	Sandwich ELISA	Competitive ELISA
Dilution Ratio	50X	2X
Rat A	25.5	414
Rat B	68.7	403
Rat C	102.9	241
Measurement Range	0.125 - 8.0 ng/ml	9.4 - 600 ng/ml
Estimated target molecule	Glu-Type Osteocalcin with Positions 21 - 50	All osteocalcin with Glu 21/24

Results:

Measurements with MK146 tend to be below those with Cat. #MK122. This may result from differences in the type of target molecule, of antibody used with the kits, and the differences in the systems and sensitivities of the 2-antibody sandwich ELISA method and the 1-antibody competitive ELISA method.

9. Simultaneous Measurement of Glu-OC and Gla-OC in Rat Serum

Both types of osteocalcin (Glu- (inactive) type and Gla- (active) type) were monitored in the blood serum of young or aged rats over several weeks.

The values in the table are reduced concentrations multiplied by the dilution ratio.

	MK126					MK146				
	Rat Gla-OC EIA kit					Rat Glu-OC EIA kit				
Age (Weeks)	Dilution	Male No. 1	Male No. 2	Female No. 1	Female No. 2	Dilution	Male No. 1	Male No. 2	Female No. 1	Female No. 2
3	x 20	12.4	—	231.5	—	x 20	0.9	—	25.1	—
4	x 60	275.2	—	1389.2	—	x 20	33.6	—	62.0	—
5	x 60	277.6	—	375.3	—	x 20	40.1	—	48.2	—
6	x 60	445.0	380.6	143.7	372.5	x 20	71.8	52.2	20.1	43.1
7	x 60	235.3	357.0	178.3	494.6	x 20	40.0	52.5	26.0	56.5
8	x 60	351.4	356.2	530.1	840.2	x 20	64.5	48.0	61.4	74.5
9	x 60	180.5	272.1	301.9	154.0	x 20	28.5	43.4	44.0	23.2

Age (Weeks)	Dilution			Female No. R1	Female No. R2	Dilution			Female No. R1	Female No. R2
21	x 30			199.7	79.8	x 10			10.2	13.3
25	x 10			52.3	55.2	x 10			5.3	5.7
29	x 10			9.1	29.5	x 10			1.6	3.2
33	x 10			80.9	38.8	x 10			7.8	4.0

Units: ng/ml

Results:

Both Gla and Glu osteocalcin in sera tend to be higher than the aged rats suggesting activation of bone metabolic turnover.

IX. Related Products

Rat Gla-Osteocalcin High Sensitive EIA Kit (Cat. #MK126)
TRACP & ALP double-stain Kit (Cat. #MK300)
TRACP & ALP Assay Kit (Cat. #MK301)
Osteoblast differentiation reagent: Osteoblast-inducer Reagent (for animal cell) (Cat. #MK430)
Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021)

X. References

1) Price, P. A. *et al.* (1980) *J Biol Chem.* **255** (7), 2938-2942.
2) Patterson-Allen, P. *et al.* (1982) *Anal Biochem.* **120**, 1-7.
3) J. Y. Fu *et al.* (1999) *Calcif Tissue Int*. **64**, 229-233.
4) Koyama, N. (1991) *J Immuno Meth.* **139**, 17-23.
5) Vergnaud, P. (1997) *J Clin Endocrinol Metab*. **82** (3), 719-724.

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.