DRG® Osteocalcin (1-43/49) human ELISA (EIA-5504)

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This kit is intended for Research Use Only.

Not for use in diagnostic procedures.

Please use only the valid version of the package insert provided with the kit.

1 INTENDED USE

This ELISA (enzyme-linked immunosorbent assay) kit is intended for measurement of both human osteocalcin (1-49) and osteocalcin (1-43) (also referred as N-terminal & mid-regional osteocalcin) levels in test samples. This test is useful for assessing the bone formation activity or osteoblast activity in individuals associated with changes in the rate of bone turnover in metabolic bone disease, such as osteoporosis, primary hyperparathyroidism, hyperthyroidism, Paget's disease, and renal osteodystrophy.

2 ASSAY PRINCIPLE

This ELISA is designed, developed and produced for measurement of human osteocalcin (1-49) and (1-43) in serum or plasma sample.

The assay utilizes the two-site "sandwich" technique with two selected antibodies that bind to different epitopes of human osteocalcin.

Assay standards, controls and specimen samples are added directly to wells of a microtiter plate that is coated with streptavidin. Subsequently, a mixture of biotinylated human osteocalcin N-terminal region specific polyclonal antibody and a peroxidase-labeled human osteocalcin 20 – 43 region specific monoclonal antibody is added to each well. After the first incubation period, a "sandwich" of "biotinylated antibody – human osteocalcin – HRP-monoclonal antibody" is formed and this immunocomplex is also captured to the wall of microtiter plate via a streptavidin-biotin affinity binding. The unbound monoclonal antibodies and buffer matrix are removed in the subsequent washing step. A substrate solution in a timed reaction is then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to the wall of each microtiter well is directly proportional to the amount of human osteocalcin in a test sample. A standard curve is generated by plotting the absorbance versus the respective human osteocalcin concentration for each standard on point-to-point or 4 parameter curve fit. The concentration of human osteocalcin in test samples is determined directly from this standard curve.

3 REAGENTS - PREPARATION AND STORAGE

This test kit must be stored at 2 °C - 8 °C upon receipt. For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

Prior to use allow all reagents to come to room temperature.

Reagents from different kit lot numbers should not be combined or interchanged.

1. Streptavidin Coated Microplate

One microplate with 12 x eight strips (96 wells total) coated with streptavidin. The plate is framed and sealed in a foil zipper bag with a desiccant.
This reagent should be stored at 2 °C - 8 °C and is stable until the expiration date on the kit box.

2. HRP-Conjugated Osteocalcin Antibody

One vial contains **1.2 mL** HRP conjugated monoclonal anti-human osteocalcin (20- 43) antibody in a stabilized protein matrix. This reagent must be diluted with biotinylated antibody before use.
This reagent should be stored at 2 °C - 8 °C and is stable until the expiration date on the kit box.

3. Biotinylated Osteocalcin Antibody

Two bottles each contains **12 mL** biotinylated anti-human osteocalcin N-terminal region specific antibody in a stabilized protein matrix.
This reagent is ready to be used for dilution of HRP conjugated osteocalcin antibody.
This reagent should be stored at 2 °C - 8 °C and is stable until the expiration date on the kit box.

4. ELISA Wash Concentrate

One bottle contains **30 mL** of 30-fold concentrate.
Before use the contents must be diluted with 870 mL of distilled water and mixed well. Upon dilution this yields a working wash solution containing a surfactant in phosphate-buffered saline with a non-azide, non-mercury preservative.
The diluted wash solution may be stored at room temperature and is stable until the expiration date on the kit box.

5. ELISA HRP Substrate

One bottle contains **22 mL** of tetramethylbenzidine (TMB) with hydrogen peroxide.
This reagent should be stored at 2 °C - 8 °C and is stable until the expiration date on the kit box.

6. ELISA Stop Solution

One bottle contains **12 mL** of sulfuric acid.
This reagent may be stored at 2 °C - 8 °C or room temperature and is stable until the expiration date on the kit box.

7. Human Osteocalcin Standards

Six vials each contain human osteocalcin in a lyophilized bovine serum-based matrix with a non-azide, non-mercury preservative.
Refer to vials for exact concentration for each standard.
These reagents should be stored at 2 °C - 8 °C and are stable until the expiration date on the kit box.

8. Human Osteocalcin Controls

Two vials each contains human osteocalcin in a lyophilized bovine serum based matrix with a non-azide, non-mercury preservative.
Refer to vials for exact concentration range for each control.
Both controls should be stored at 2 °C - 8 °C and are stable until the expiration date on the kit box.

4 SAFETY PRECAUTIONS

The reagents must be used in professional laboratory.

Source material for reagents containing bovine serum was derived in the contiguous 48 United States. It was obtained only from healthy donor animals maintained under veterinary supervision and found free of contagious diseases.

Wear gloves while performing this assay and handle these reagents as if they are potentially infectious.

Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. TMB is a suspected carcinogen. Sulfuric acid may cause severe irritation on contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes.

Use Good Laboratory Practices.

5 MATERIALS REQUIRED BUT NOT PROVIDED

1. Serum or plasma sample collection tube.
2. Precision single channel pipettes capable of delivering 25 µL, 100 µL, 200 µL, and 1000 µL etc.
3. Repeating dispenser suitable for delivering 100 µL and 200 µL.
4. Disposable pipette tips suitable for above volume dispensing.
5. Disposable 12 x 75 mm or 13 x 100 plastic test tubes.
6. Disposable plastic 1000 mL bottle with cap.
7. Aluminum foil.
8. Deionized or distilled water.
9. Plastic microtiter well cover or polyethylene film.
10. ELISA multichannel wash bottle or automatic (semi-automatic) washing system.
11. Spectrophotometric microplate reader capable of reading absorbance at 450 nm.
12. ELISA plate shaker.

6 SPECIMEN COLLECTION

Only 50 µL of human serum or plasma sample is required for human osteocalcin measurement in duplicate.

No special preparation of individual is necessary prior to specimen collection.

Whole blood should be collected and must be allowed to clot for minimum 30 minutes at room temperature before the serum is separated by centrifugation (850 – 1500 xg for 10 minutes). The serum should be separated from the clot within three hours of blood collection and transferred to a clean test tube.

Serum sample is allowed to be stored at 2 °C - 8 °C or room temperature for 6 days until measurement.

Sample should be stored in frozen condition (< -20 °C) for longer storage.

Avoid more than three freeze-thaw cycles of specimen. It is necessary to take care in the sample collection procedure to avoid haemolysis.

7 ASSAY PROCEDURE

7.1 Reagent Preparation

1. Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.
2. ELISA Wash Concentrate must be diluted to working solution prior use.
   Please see REAGENTS section for details.
3. Reconstitute all assay standards and controls by adding **0.5 mL** of distilled or deminerialized water to each vial.
   Allow the standards and controls to sit undisturbed for 5 minutes, and then mix well by inversions or gentle vortexing. Make sure that all solid is dissolved completely prior to use.
   These reconstituted standards and controls must be stored at -18 °C or below.
   Do not exceed 3 freeze-thaw cycles.

7.2 Assay Procedure

1. Place a sufficient number of streptavidin coated microwell strips in a holder to run human osteocalcin standards, controls and unknown samples in duplicate. The unused strips should be resealed in the bag with a desiccant and stored at 2 °C - 8 °C.
2. Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3
A	STD 1	STD 5	SAMPLE 1
B	STD 1	STD 5	SAMPLE 1
C	STD 2	STD 6	SAMPLE 2
D	STD 2	STD 6	SAMPLE 2
E	STD 3	C 1	SAMPLE 3
F	STD 3	C 1	SAMPLE 3
G	STD 4	C 2	
H	STD 4	C 2	

3. Prepare working HRP conjugated Osteocalcin Antibody and Biotinylated Osteocalcin Antibody by **1:21 fold** dilution of the conjugation antibody with the biotinylated antibody solution.
Following is a table that outlines the relationship of strips used and antibody mix prepared.

Strip no.	Biotinylated Antibody Solution	HRP-Conjugated Antibody
1	2 mL	100 µL
2	4 mL	200 µL
3	6 mL	300 µL
4	8 mL	400 µL
5	10 mL	500 µL
6	12 mL	600 µL
7	14 mL	700 µL
8	16 mL	800 µL
9	18 mL	900 µL
10	20 mL	1000 µL
11	22 mL	1100 µL
12	24 mL	1200 µL

Note: this antibody mixture should be freshly prepared.

4. Add **25 µL** of standards, controls and specimen serum/plasma samples into the designated microwell.
5. Add **200 µL** of above antibody mixture to each well
6. Cover the plate with one plate sealer and also with aluminum foil to avoid exposure to light.
7. Incubate the plate at room temperature, shaking 350 rpm ± 100 rpm for **1 hour**
8. Remove the aluminum foil and plate sealer. Aspirate the contents of each well. Wash each well 5 times by dispensing 350 µL - 400 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
9. Add **200 µL** of ELISA HRP Substrate into each of the wells.
10. Cover the plate with one new plate sealer and also with aluminum foil to avoid exposure to light.
11. Incubate plate at room temperature static for **20 minutes** *(This incubation period may be reduced to 8 - 15 min if a lower OD reading is demanded to fit to the plate readers specification)*
12. Remove the aluminum foil and plate sealer.
Add **50 µL** of ELISA Stop Solution into each of the wells. Mix gently.
13. Read the absorbance at **450 nm** within 10 minutes in a microplate reader

NOTE: in case extremely low background is required, one can set the instrument to dual wavelength measurement at 450 nm with background wavelength correction set at 595 nm, 620 nm or 630 nm.

8 PROCEDURAL NOTES

14. It is recommended that all standards, controls and unknown samples be assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results.
15. Keep light-sensitive reagents in the original amber bottles.
16. Store any unused streptavidin-coated strips in the foil zipper bag with desiccant to protect from moisture.
17. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
18. Incubation times or temperatures other than those stated in this insert may affect the results.
19. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading.
20. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.
21. Prepare a calibration curve for each run. Do not use data from previous runs.
22. To avoid cross-contamination, use a clean disposable pipette tip for the addition of each reagent and sample.

9 INTERPRETATION OF RESULTS

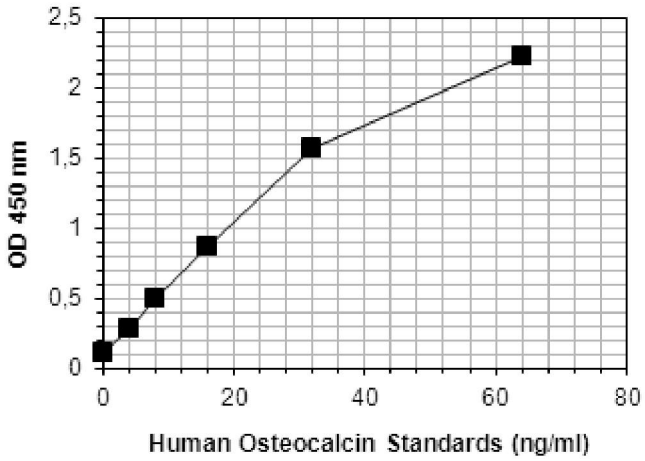
1. Calculate the average absorbance for each pair of duplicate test results.
2. Subtract the average absorbance of the zero standard from the average absorbance of all other readings to obtain corrected absorbance.
3. The standard curve is generated by the corrected absorbance of all standard levels on the ordinate against the standard concentration on the abscissa using point-to-point or log-log paper. Appropriate computer assisted data reduction programs (e.g. Point-to-Point, 4-Parameter) may also be used for the calculation of results.

The sample human osteocalcin concentrations for the controls and the specimen samples are read directly from the standard curve using their respective corrected absorbance.

10 EXAMPLE DATA AND STANDARD CURVE

A typical absorbance data and the resulting standard curve from this human osteocalcin ELISA are represented. **This curve should not be used in lieu of standard curve run with each assay.**

Well I.D.	Results		
	Average	Corrected	
0 ng/mL	0.112	0.000	
4 ng/mL	0.279	0.167	
8 ng/mL	0.494	0.382	
16 ng/mL	0.866	0.754	
32 ng/mL	1.570	1.458	
64 ng/mL	2.232	2.120	
Control 1	0.363	0.251	5.26 ng/mL
Control 2	0.663	0.551	11.69 ng/mL



11 QUALITY CONTROL

To assure the validity of the results each assay should include adequate controls.

12 REFERENCES

1. Rosenquist C, Qvist P, Bjarnason N, Christiansen C. Measurement of a more stable region of osteocalcin in serum by ELISA with two monoclonal antibodies. Clin Chem. 1995 Oct;41(10):1439-45.
2. Takahashi M, Kushida K, Nagano A, Inoue T. Comparison of the analytical and clinical performance characteristics of an N-MID versus an intact osteocalcin immunoradiometric assay. Clin Chim Acta. 2000 Apr;294(1-2):67-76.
3. Nagasue K, Inaba M, Okuno S, Kitatani K, Imanishi Y, Ishimura E, Miki T, Kim M, Nishizawa Y. Serum N-terminal midfragment vs. intact osteocalcin immunoradiometric assay as markers for bone turnover and bone loss in hemodialysis patients. Biomed Pharmacother. 2003 Mar;57(2):98-104.
4. Garnero P, Grimaux M, Seguin P, Delmas PD. Characterization of immunoreactive forms of human osteocalcin generated in vivo and in vitro. J Bone Miner Res. 1994 Feb;9(2):255-64

13 SHORT ASSAY PROCEDURE

4. Add 25 µL of standards, controls and specimen samples into the designated microwell.
5. Add 200 µL of antibody mixture to each well.
6. Incubate 60 minutes at RT, shaking 350 rpm.
7. Wash each well 5 times.
8. Add 200 µL of ELISA HRP Substrate into each of the wells.
9. Cover and incubate plate at room temperature static for 20 minutes.
10. Add 50 µL of ELISA Stop Solution into each of the wells.
11. Read the absorbance at 450 nm.

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