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# ab270202
# Human Osteocalcin SimpleStep ELISA® Kit

For the quantitative measurement of Osteocalcin in human serum, plasma and cell culture supernatant samples.

This product is for research use only and is not intended for diagnostic use.

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# 1. Overview

Osteocalcin *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of Osteocalcin protein in human serum, plasma and cell culture supernatant samples

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB Development Solution is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

Osteocalcin is a small secreted protein 49 amino acids produced by osteoblasts. Osteocalcin acts as a hormone impacting bone mineral absorption, glucose metabolism and insulin secretion. During bone reabsorption osteoclasts break down the bone matrix releasing bound Osteocalcin into 5 different fragments; N-terminal, Mid, C-terminal, N-terminal-Mid, and Mid-C-terminal into the serum. Circulating levels of intact Osteocalcin and Osteocalcin fragments have been found to vary significantly between healthy individuals and diseased stats such as Paget's disease, osteoporosis, fracture, and renal failure. The standard peptide in this product is mature Osteocalcin.

## 2. Protocol Summary

Prepare all reagents, samples, and standards as instructed

Add 50 µL standard or sample to appropriate wells

Add 50 µL Antibody Cocktail to all wells

Incubate at room temperature for 1 hour

Aspirate and wash each well three times with 350 µL 1X Wash Buffer PT

Add 100 µL TMB Development Solution to each well and incubate for 10 minutes.

Add 100 µL Stop Solution and read OD at 450 nm

## 3. Precautions

**Please read these instructions carefully prior to beginning the assay.**

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn.
- If applicable, please refer to the current Safety Data Sheet (SDS) provided with this product for safety, handling, and disposal information. The most up to date and current versions are available on our website https://www.abcam.com/en-us.

## 4. Storage and Stability

**Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.**

Refer to list of materials supplied for storage conditions of individual components.

## 5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

## 6. Materials Supplied

| Item | Quantity | Storage Condition |
|---|---|---|
| Human Osteocalcin Capture Antibody 10X | 600 µL | +4°C |
| Human Osteocalcin Detector Antibody 10X | 600 µL | +4°C |
| Human Osteocalcin Lyophilized Peptide | 2 Vials | +4°C |
| Antibody Diluent 4BI | 6 mL | +4°C |
| Sample Diluent NS | 12 mL | +4°C |
| Wash Buffer PT 10X | 20 mL | +4°C |
| TMB Development Solution | 12 mL | +4°C |
| Stop Solution | 12 mL | +4°C |
| SimpleStep Pre-Coated 96-Well Microplate | 96 Wells | +4°C |
| Plate Seal | 1 | +4°C |

## 7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

## 8. Technical Hints

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.

- **To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.**
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

## 9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

### 9.1 1X Wash Buffer PT:

Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

### 9.2 Antibody Cocktail:

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 4BI. To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent 4BI. Mix thoroughly and gently.

## 10. Standard Preparation

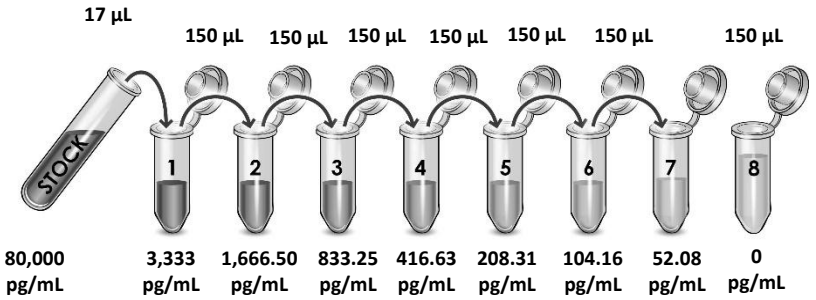
- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

**10.1** **IMPORTANT:** If the protein standard vial has a volume identified on the label, reconstitute the Osteocalcin by adding that volume of Sample Diluent NS indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the Osteocalcin standard by adding 500 µL of Sample Diluent NS. Hold at room temperature for 10 minutes and mix gently. This is the 80 ng/mL **Stock Standard** Solution.

10.1.1 Label eight tubes, Standards 1– 8.

10.1.2 Add 391 µL Sample Diluent NS into tube number 1 and 150 µL of Sample Diluent NS into numbers 2-8.

10.1.3 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:



## 11. Sample Preparation

| Typical Sample Dynamic Range | |
|---|---|
| **Sample Type** | **Range** |
| Serum | 0.23 – 7.5% |
| Plasma - Citrate | 0.63 – 10% |
| Plasma - Heparin | 0.47 – 7.5% |
| Plasma - EDTA | 0.31 – 10% |
| 1640 RPMI Cell Culture Media* | ≤ 50% |

*Based on spiked sample.

### 11.1 Serum:

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples at least 1: 13.3 into Sample Diluent NS and assay. Store un-diluted serum at -20ºC or below. Avoid repeated freeze-thaw cycles.

### 11.2 Plasma:

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute heparin samples at least 1: 13.3 and citrate and EDTA samples at least 1: 10 into Sample Diluent NS and assay. Store un-diluted plasma samples at -20ºC or below for up to 3 months. Avoid repeated freeze-thaw cycles.

### 11.3 Cell Culture Supernatants:

Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants and assay. Or dilute samples at least 1: 2 into Sample Diluent NS and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles. Please note that supernatants containing fetal bovine serum are not recommended for use in this kit.

## 12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or "edge effects" have not been observed with this assay.

## 13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.

**13.1** Prepare all reagents, working standards, and samples as directed in the previous sections.

**13.2** Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

**13.3** Add 50 µL of all sample or standard to appropriate wells.

**13.4** Add 50 µL of the Antibody Cocktail to each well.

**13.5** Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.

**13.6** Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Wash Buffer PT should remain in wells for at least 10 seconds. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and tap gently against clean paper towels to remove excess liquid.

**13.7** Add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.
*Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.*
*Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.*

**13.8** Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

**13.9** Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed

time in the microplate reader prepared with the following settings:

| Mode | Kinetic |
|---|---|
| Wavelength: | 600 nm |
| Time: | up to 20 min |
| Interval: | 20 sec - 1 min |
| Shaking: | Shake between readings |

Δ **Note**: that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450 nm.

**13.10** Analyze the data as described below.

## 14. Calculations

**14.1** Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.

**14.2** **Create a standard curve** by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.

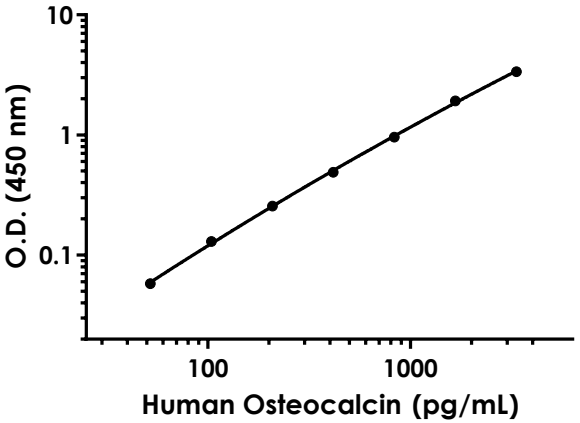
Δ **Note:** Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.

**14.3** Determine the concentration of the target protein in the sample by interpolating the blank control subtracted **absorbance values against the standard curve**. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.

**14.4** Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

## 15. Typical Data

**Typical standard curve** – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.




| Standard Curve Measurements | | | |
|---|---|---|---|
| Concentration (pg/mL) | O.D 450 nm | | Mean O.D |
| | 1 | 2 | |
| 0 | 0.048 | 0.046 | 0.047 |
| 52.08 | 0.107 | 0.107 | 0.107 |
| 104.16 | 0.179 | 0.177 | 0.178 |
| 208.31 | 0.305 | 0.305 | 0.305 |
| 416.63 | 0.526 | 0.550 | 0.538 |
| 833.25 | 0.977 | 1.041 | 1.009 |
| 1,666.50 | 1.944 | 2.003 | 1.973 |
| 3,333 | 3.357 | 3.473 | 3.415 |

**Figure 1**. Example of human Osteocalcin standard curve in Sample Diluent NS. The Osteocalcin standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

## 16. Typical Sample Values

**SENSITIVITY** –

The calculated minimal detectable dose (MDD) is 13.99 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=16) and adding 2 standard deviations then extrapolating the corresponding concentration.

**RECOVERY** –

Three concentrations of Osteocalcin synthetic standard peptide were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

| Sample Type | Average % Recovery | Range (%) |
|---|---|---|
| Serum (1%) | 93 | 80 – 103 |
| Plasma – Citrate (1%) | 90 | 84 – 97 |
| Plasma – Heparin (1%) | 93 | 81 – 99 |
| Plasma – EDTA (1%) | 93 | 90 – 96 |
| Cell Culture Media (50%)* | 102 | 91 – 115 |

*Media is RPMI 1640. Media containing fetal bovine serum is not compatible with this kit.

**Linearity of Dilution**

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Native Osteocalcin was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

| Dilution Factor | Interpolated value | 7.5% Human Serum | 10% Human Plasma (Citrate) | 7.5% Human Plasma (Heparin) | 10% Human Plasma (EDTA) |
|---|---|---|---|---|---|
| Undiluted | pg/mL | 2,666 | 2,422 | 2,628 | 3,217 |
| | **% Expected value** | 100 | 100 | 100 | 100 |
| 2 | pg/mL | 1,307 | 1,151 | 1,552 | 1,563 |
| | **% Expected value** | 98 | 95 | 118 | 97 |
| 4 | pg/mL | 610 | 571 | 754 | 757 |
| | **% Expected value** | 92 | 94 | 115 | 94 |
| 8 | pg/mL | 308 | 286 | 357 | 380 |
| | **% Expected value** | 92 | 94 | 109 | 95 |
| 16 | pg/mL | 172 | 157 | 204 | 207 |
| | **% Expected value** | 103 | 104 | 124 | 103 |

Recombinant Osteocalcin was spiked into the following biological samples and diluted in a 2-fold dilution series in Sample Diluent NS.

| Dilution Factor | Interpolated value | 50% 1640 RPMI Media |
|---|---|---|
| Undiluted | pg/mL | 1,713 |
| | **% Expected value** | 100 |
| 2 | pg/mL | 863 |
| | **% Expected value** | 101 |
| 4 | pg/mL | 426 |
| | **% Expected value** | 99 |
| 8 | pg/mL | 204 |
| | **% Expected value** | 95 |
| 16 | pg/mL | 118 |
| | **% Expected value** | 110 |

**PRECISION** –

Mean coefficient of variations of interpolated values of Osteocalcin from two concentrations of plasma (EDTA) within the working range of the assay.

| | Intra-Assay | Inter-Assay |
|---|---|---|
| n = | 8 | 3 |
| CV(%) | 6.1 | 7.0 |

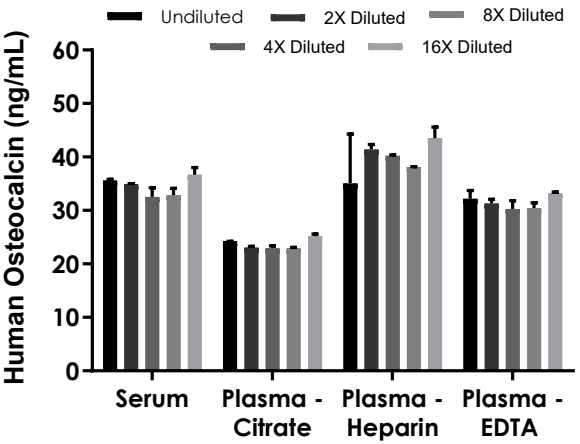



**Figure 2.** Interpolated concentrations of native Osteocalcin in human serum and plasma samples. The concentrations of Osteocalcin were measured in duplicates, interpolated from the Osteocalcin standard curves and corrected for sample dilution. Undiluted samples are as follows: serum 7.5%, plasma (citrate) 10%, plasma (heparin) 7.5%, and plasma (EDTA) 10%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean Osteocalcin concentration was determined to be 34.49 ng/mL in serum, 24.94 ng/mL in plasma (citrate), 41.51 ng/ml in plasma (heparin), and 32.69 ng/ml in plasma (EDTA).

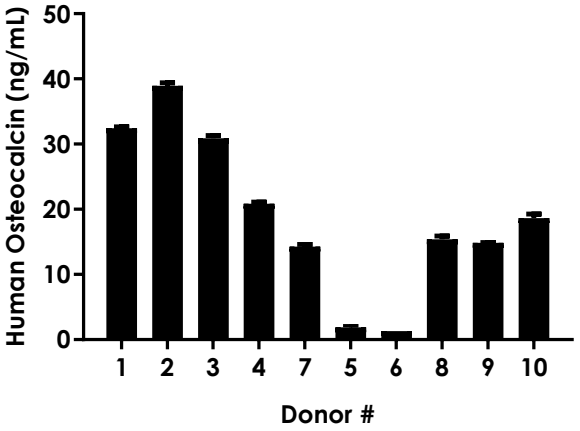



**Figure 3.** Serum from ten individual healthy human female donors was measured in duplicate. Interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean Osteocalcin concentration was determined to be 18.95 ng/mL with a range of 1.30 – 38.94 ng/mL.

## 17. Assay Specificity

This kit recognizes both native and recombinant human Osteocalcin protein in serum, plasma, and cell and tissue culture supernatant samples only.

Cell and tissue extract samples have not been tested with this kit.

This kit is incompatible with supernatant samples made with fetal bovine serum due to cross reactivity.

## 18. Species Reactivity

This kit recognizes human Osteocalcin protein.

Other species reactivity was determined by measuring 5% mouse, rat, and bovine serum samples, interpolating the Osteocalcin protein concentrations from the human standard curve, and expressing the interpolated concentrations as a percentage of the Osteocalcin protein concentration in human serum assayed at the same dilution.

Reactivity < 3% was determined for the following species:

- Mouse
- Rat
- Cow

Normal bovine serum cross reactivity was not observed; however, significant fetal bovine serum cross reactivity was observed starting at a concentration of 0.5% or higher. We recommend serum-free cell culture media or that the user test their fetal bovine serum for signal with this product.

Please contact our Technical Support team for more information.

## 19. Troubleshooting

| Problem | Reason | Solution |
|---|---|---|
| **Poor standard curve** | Inaccurate Pipetting | Check pipettes |
| | Improper standard dilution | Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing |
| **Low Signal** | Incubation times too brief | Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation |
| | Inadequate reagent volumes or improper dilution | Check pipettes and ensure correct preparation |
| | Incubation times with TMB too brief | Ensure sufficient incubation time until blue color develops prior addition of Stop solution |
| **Large CV** | Plate is insufficiently washed | Review manual for proper wash technique. If using a plate washer, check all ports for obstructions. |
| | Contaminated wash buffer | Prepare fresh wash buffer |
| **Low sensitivity** | Improper storage of the ELISA kit | Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB Development Solution protected from light. |
| **Precipitate in Diluent** | Precipitation and/or coagulation of components within the Diluent. | Precipitate can be removed by gently warming the Diluent to 37°C. |

## 20. Notes

**Technical Support**

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