HUMAN OSTEOPONTIN

Immunoperoxidase Assay for Determination of Osteopontin in Human Samples

DIRECTIONS FOR USE

Version3 L25.0—2Q1

For Research Use Only, NOT for Diagnostic Purposes

Please Read this Package Insert Completely Before Using This Product

INTENDED USE

The Human Osteopontin ELISA is designed for the quantitative determination of osteopontin in human plasma and urine.

PRINCIPLE OF THE ASSAY

The Human Osteopontin ELISA is a sandwich type immunoassay. The 96-well microplate is coated with a polyclonal antibody specific for human osteopontin. The standards, controls, and samples are added to the microplate wells. The microplate is then incubated at room temperature without agitation. After the first incubation is complete, the wells are washed with Wash Buffer and blotted dry. The polyclonal detection antibody conjugate is then added, and the microplate is incubated a second time at room temperature without agitation. After the second incubation is complete, the wells are washed with Wash Buffer and blotted dry. HRP conjugated streptavidin is then added, and the microplate is incubated a third time. After the third incubation is complete, the wells are washed with Wash Buffer and blotted dry. TMB Substrate is added, and the microplate is incubated a fourth time at room temperature without agitation. Once the incubation is complete, Stop Solution is added, and the optical density (OD) is measured by a spectrophotometer at 450 nm. The intensity of the color generated is directly proportional to the amount of human osteopontin in the sample.

MATERIALS SUPPLIED

Component	Quantity	Preparation
Human Osteopontin Microplate (96 wells)	12 x 8 strips	Ready to use
Diluent Buffer	50 mL	Ready to use
Human Osteopontin Control	1 vial	40X
Biotin Conjugate	0.150 mL	100X
HRP Streptavidin Conjugate	0.150 mL	100X
Wash Buffer	50 mL	20X
TMB Substrate	12 mL	Ready to use
Stop Solution	12 mL	Ready to use

*Please refer to the Certificate of Analysis enclosed with each kit for more information.

MATERIALS REQUIRED

- Precision pipettes for dispensing up to 1000 µL (with disposable tips)
- Repeating or multi-channel pipette for dispensing up to 1000 µL
- Volumetric containers and pipettes for reagent preparation
- Distilled or deionized water for reagent preparation
- Microplate washer or wash bottle
- Microplate reader with 450 nm filter
- Vortex for sample preparation
- Centrifuge (10000 – 20000 rpm) for sample preparation

PRECAUTIONS

1. The human blood products incorporated into this kit have been tested for the presence of HIV (Human Immunodeficiency virus), HBV (Hepatitis B virus), and HCV (Hepatitis C virus). Test methods for these viruses do not guarantee the absence of a virus; therefore, all reagents should be treated as potentially infectious. Handling and disposal should be in accordance with all appropriate national and local regulations for the handling of potentially biohazardous materials.
2. All materials derived from animal sources are BSE negative. However, all materials should be kept from ruminating animals.
3. Avoid direct contact with skin.
4. This product is not for internal use.
5. Avoid eating, drinking, or smoking when using this product.
6. Do not pipette any reagents by mouth.
7. Reagents from this kit are lot-specific and must not be substituted.
8. Do not use reagents beyond the expiration date.
9. Variations to the test procedure are not recommended and may influence the test results.

STORAGE CONDITIONS

The kit should be stored at 2-8°C with the exception of the calibrator. The kit is stable until the expiration date on the box label.

SAMPLE HANDLING AND PREPARATION

Human plasma and urine samples are appropriate for use in this assay.

Human serum samples are not recommended for use because of a proteolytic cleavage, by thrombin, during clotting processes. OD values obtained from measuring human serum samples are expected to be lower than those measured in human plasma samples.

All samples should be centrifuged for 10 minutes at 13,000 rpm, and the supernatant used in the assay. Discard any remaining sample.

It is recommended that all samples are assayed in duplicate. Dilute samples with 1X Diluent Buffer and gently vortex. For duplicate samples, dilute 10 µL of sample in 390 µL with 1X Diluent Buffer. If a sample has a concentration of human osteopontin greater than the highest standard, dilute the sample further in 1X Diluent Buffer and repeat the analysis

The following sample dilutions are recommendations and should be optimized for each laboratory:

1. Human plasma – 1:40 dilution
2. Human urine – 1:100 dilution

It is recommended to 1) thoroughly vortex each sample before use and 2) perform pipetting actions without pausing.

REAGENT PREPARATION

All reagents must be equilibrated to room temperature prior to preparation and subsequent use in the assay.

20X Wash Buffer is to be diluted 20-fold with deionized water. For example, to prepare Working Strength Wash Buffer, dilute 20 mL of Wash Buffer Concentrate (20X) with 380 mL of deionized water. Working Strength Wash Buffer is stable for 4 weeks at room temperature (18-25°C).

Calibrator is provided in a liquid form at a stock concentration. Please refer to the lot-specific Certificate of Analysis for actual concentration. The stock calibrator should be stored at ≤ - 10°C. If multiple assays are to be performed, stock calibrator aliquots should be store at ≤ -10°C to minimize freeze thaw cycles.

Prepare dilution series using the stock Calibrator and 1X Diluent Buffer as in the following example. ***Actual Calibrator and Standard concentrations are provided on the lot-specific Certificate of Analysis that is provided with each kit.*** Unused diluted calibrator should be discarded after use.

Standard	Volume of Calibrator/Standard	Volume of 1X Diluent Buffer	Final Concentration (ng/ml)
7	10 µl Stock Calibrator	730 µl	20
6	500 µl Standard 7	500 µl	10
5	500 µl Standard 6	500 µl	5.0
4	500 µl Standard 5	500 µl	2.5
3	500 µl Standard 4	500 µl	1.25
2	500 µl Standard 3	500 µl	0.625
1	500 µl Standard 2	500 µl	0.3125
0	N/A	500 µl	0

Biotin Conjugate is to be diluted 100-fold with 1X Diluent Buffer. For example, to prepare enough Working Strength Biotin Conjugate for one complete microplate, dilute 0.1 mL of Biotin Conjugate (100X) with 9.9 mL of 1X Diluent Buffer. The Working Strength Biotin Conjugate is stable for up to 1 hour when stored in the dark at room temperature.

HRP-SA Conjugate is to be diluted 100-fold with 1X Diluent Buffer. For example, to prepare enough Working Strength HRP-SA Conjugate for one complete microplate, dilute 0.1 mL of HRP-Streptavidin Conjugate (100X) with 9.9 mL of 1X Diluent Buffer. The Working Strength HRP-SA Conjugate solution is stable for up to 1 hour when stored in the dark at room temperature.

ASSAY PROCEDURE

Document for Reference Use Only. Refer to Document shipped with Product to run Assay.

All reagents and microplate strips should be equilibrated to room temperature (18-25°C) for at least 30 minutes before use. Gently mix all reagents before use. A standard curve must be performed with each assay run and with each microplate, if more than one plate is used at a time. All standards, controls, and samples should be run in duplicate.

1. The microplate should be equilibrated to room temperature prior to opening the foil pouch. Designate enough microplate strips for duplicate determinations of the standards, controls, and samples. The remaining microplate strips should be stored at 2-8°C in the tightly sealed foil pouch containing the desiccant.

2. **Pipette 100 µL** of each standard and diluted sample into their respective wells. See *Reagent Preparation* and Certificate of Analysis for standard, control and sample dilution instructions.

3. Cover microplate with a plate sealer and **incubate for 2 hours** at room temperature. Keep the plate level during the incubation

4. Decant the contents of the wells and **wash the microplate 4 times** with 350 µL of Working Strength Wash Buffer per well (see *Reagent Preparation*) using a microplate washer. Alternatively, fill the wells with Working Strength Wash Buffer using a wash bottle equipped with a wash nozzle. (It is not recommended to use a multichannel pipette. Wash buffer must be dispensed with adequate and equal force in order to properly wash the wells.) Between washes, invert the microplate to discard the liquid and firmly tap the inverted microplate on absorbent paper towels. After the final wash, (automated or manual), remove any residual Wash Buffer and bubbles from the wells by inverting and firmly tapping the microplate on absorbent paper towels.

5. **Pipette 100 µL** of Working Strength Biotin Conjugate into each well (*See Reagent Preparation)*.

6. Cover microplate with a plate sealer and **incubate in the dark for 20 minutes** at room temperature. Keep the plate level during the incubation.

7. Decant the contents of the wells and **wash the microplate 4 times** with 350 µL of Working Strength Wash Buffer as per washing method described in Step 4.

8. **Pipette 100 µL** of Working Strength HRP-SA Conjugate into each well (*See Reagent Preparation)*.

9. Cover microplate with a plate sealer and **incubate in the dark for 20 minutes** at room temperature. Keep the plate level during the incubation.

10. Decant the contents of the wells and **wash the microplate 4 times** with 350 µL of Working Strength Wash Buffer as per washing method described in Step 4.

11. **Pipette 100 µL** of TMB Substrate into each well.

12. **Incubate the plate in the dark for 10 minutes** at room temperature. Keep the plate level during the incubation.

13. **Pipette 100 µL** of Stop Solution into each well and gently shake the microplate to mix the contents. Remove any bubbles before proceeding with the next step.

14. Place the microplate in a microplate reader capable of reading the absorbance at 450 nm. The microplate should be analyzed immediately after the addition of the Stop Solution, and no longer than 30 minutes after.

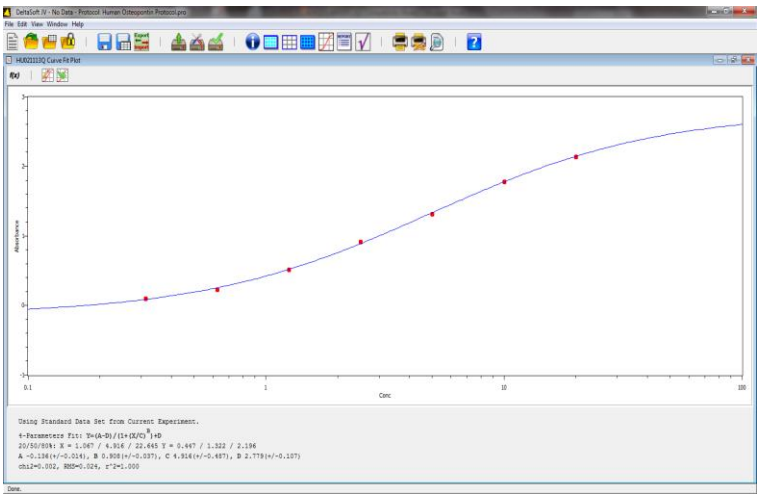
CALCULATION OF RESULTS

Construct a standard curve from the standards. It is recommended to use a software program to calculate the standard curve and to determine the concentration of the samples. It is recommended to use a 4 parameter logistic (log-log) curve fit, utilizing $1/y^2$ weighting, for this assay. If samples were diluted, the appropriate dilution factor should be used to calculate the actual sample values.

TYPICAL STANDARD CURVE

The following results are provided for demonstration purposes only and cannot be used in place of data obtained with the assay. A standard curve must be performed with each assay run and plate tested.

Standards (ng/mL)	Absorbance (450 nm)
20	2.24
10	1.88
5.0	1.41
2.5	1.01
1.3	0.615
0.63	0.328
0.31	0.196
0	0.098



PERFORMANCE CHARACTERISTICS

Sensitivity
The analytical sensitivity (Lower Limit of Detection) was determined by calculating the mean + 2.5 standard deviations for 20 replicates of the Zero Standard. The analytical sensitivity of the assay is 0.050 ng/mL.

The functional sensitivity is defined as the lowest concentration which exhibits accuracy between 80% - 120% and precision < 20%. 16 replicates were evaluated at varying known concentrations of human osteopontin. The functional sensitivity of the assay is 0.31 ng/mL

Precision: Within run (intra-assay) variation
The within run precision is expressed as the percentage coefficient of variation (CV %). This was determined based on the mean and standard deviation of 12 replicates of a sample run in a single assay. The table below shows the dilution corrected results of 4 samples that span the range of the assay.

	Sample 1	Sample 2	Sample 3	Sample 4
Mean	276 ng/mL	69 ng/mL	53 ng/mL	13 ng/mL
Std. Dev.	27 ng/mL	5.5 ng/mL	2.0 ng/mL	0.70 ng/mL
CV %	9.8	7.8	3.6	5.4
n	12	12	12	12

Precision: Between run (inter-assay) variation
The between run precision is expressed as the percentage coefficient of variation (CV %). This was determined based on the mean and standard deviation across 9 assays of quadruplicate measurements of a single sample. The table below shows the dilution corrected results of 4 samples that span the range of the assay.

	Sample 1	Sample 2	Sample 3	Sample 4
Mean	284 ng/mL	68 ng/mL	53 ng/mL	12 ng/mL
Std. Dev.	11 ng/mL	2.8 ng/mL	3.2 ng/mL	0.66 ng/mL
CV %	4.2	4.2	6.1	5.2
n	36	36	36	36

Linearity

The linearity of the assay was determined by preparing dilutions of a sample with human osteopontin concentrations high enough to adequately measure in the 1X Diluent Buffer. The expected values were compared to the obtained values to determine a percent recovery. The average recovery across all samples was 99%.

	Observed (ng/mL)	Expected (ng/mL)	% Recovery
Human Plasma Pool #1			
1:20 dilution	2.4	-	-
1:40 dilution	1.2	1.2	96
1:80 dilution	0.52	0.61	86
1:160 dilution	0.26	0.30	84
Human Plasma Pool #2			
1:20 dilution	1.1	-	-
1:40 dilution	0.55	0.55	101
1:80 dilution	0.27	0.27	100
1:160 dilution	0.15	0.14	106
Human Urine #1			
1:100 dilution	2.1	-	-
1:200 dilution	1.2	1.1	110
1:400 dilution	0.57	0.53	106
Human Urine #2			
1:100 dilution	5.7	-	-
1:200 dilution	2.8	2.8	99
1:400 dilution	1.4	1.4	101

Spike and Recovery

The spike and recovery of the assay was determined by adding various known amounts of human osteopontin to a sample. This spiked sample was evaluated in the assay and the measured concentration was compared to the expected concentration (endogenous - spiked). Across 3 samples, the range of recovery was 85 – 115 % with an average of 93 %.

Specificity

The table below indicates the analyte and the percent cross-reactivity observed in the assay.

Analyte	% Cross-reactivity
Mouse Osteopontin	< 0.1%
Canine Osteopontin	< 1%
Rat Osteopontin	< 0.5%
Human Thrombin	Not Reactive

SHORT ASSAY PROTOCOL

Total Time = 3 hours