

KAMIYA BIOMEDICAL COMPANY

Dog Osteopontin ELISA

**For the quantitative determination of Osteopontin (PONT)
in dog biological fluid**

Cat. No. KT-995

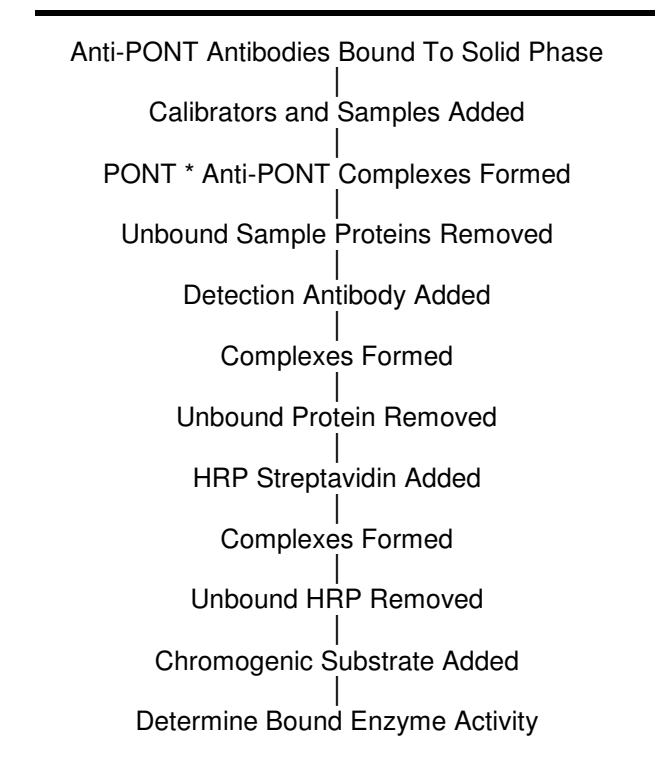
For research use only.

PRODUCT INFORMATION**Dog Osteopontin ELISA**
Cat. No. KT-995**INTENDED USE**

The Dog Osteopontin ELISA is a highly sensitive two-site enzyme-linked immunoassay (ELISA) for the quantitative determination of Osteopontin (PONT) in dog biological fluid. For research use only.

PRINCIPLE

The principle of the double antibody sandwich ELISA is represented in Figure 1. In this assay the PONT present in the sample reacts with the anti-PONT antibodies which have been adsorbed to the surface of polystyrene microtiter wells. After the removal of unbound proteins by washing, anti-PONT antibodies conjugated with horseradish peroxidase (HRP) are added. These enzyme-labeled antibodies form complexes with the previously bound PONT. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme is proportional to the concentration of PONT in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of PONT in the test sample. The quantity of PONT in the test sample can be interpolated from the calibration curve constructed from the calibrators, and corrected for sample dilution.

Figure 1.**COMPONENTS**

1. Diluent Concentrate
One bottle containing 60 mL of a 1X diluent running buffer.
2. Wash Solution Concentrate
One bottle containing 50 mL of a 20X concentrated wash solution.
3. Detection Antibody 100X
One vial containing 150 µL of affinity purified anti-Dog PONT antibody conjugated with biotin in a stabilizing buffer.

4. HRP-Streptavidin 100X
One vial containing 150 μL of HRP conjugated streptavidin in a stabilizing buffer.
5. TMB Substrate Solution
One vial containing 12 mL of TMB and hydrogen peroxide in citric acid buffer at pH 3.3.
6. Stop Solution
One vial containing 12 mL of 0.3 M sulfuric acid. **WARNING:** Avoid contact with skin.
7. Microtiter Plate
Twelve removable eight-well micro strips in well holder frame. Wells are coated with affinity-purified anti-dog PONT.
8. Dog PONT Calibrator
One vial containing a lyophilized Dog PONT Calibrator.

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes (2 μL to 200 μL) for making and dispensing dilutions
- Test tubes
- Microplate washer/aspirator
- Distilled or de-ionized H_2O
- Microplate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer

PRECAUTIONS

1. Read the instructions carefully before beginning the assay.
2. This kit is for research use only.
3. Great care has been taken to ensure the quality and reliability of this product. However, it is possible that in certain cases, unusual results may be obtained due to high levels of interfering factors.
4. No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.
5. Azide and thimerosal at concentrations higher than 0.1% inhibit the enzyme reaction.
6. Other precautions:
 - Do not interchange kit components from different lots.
 - Do not use kit components beyond the expiration date.
 - Protect reagents from direct sunlight.
 - Do not pipette by mouth.
 - Do not eat, drink, smoke or apply cosmetics where reagents are used.
 - Avoid all contact with the reagents by using gloves.
 - Stop solution contains diluted sulfuric acid. Irritation to eyes and skin is possible. Flush with water after contact.

REAGENT PREPARATION

1. Diluent
Ready to use as supplied.
2. Wash Solution Concentrate
The Wash Solution supplied is a 20X concentrate and must be diluted 1:20 with distilled or de-ionized water. Crystal formation in the concentrate is not uncommon when storage temperatures are low. Warming of the concentrate to 30-35°C before dilution can dissolve crystals.
3. Detection Antibody 100X
Calculate the required amount of working conjugate solution for each microtiter plate test strip by adding 10 μL detection antibody to 990 μL of 1X Diluent for each test strip to be used for testing. Mix uniformly, but gently. Avoid foaming.
4. HRP-Streptavidin 100X
Calculate the required amount of working conjugate solution for each microtiter plate test strip by adding 10 μL HRP-Streptavidin to 990 μL of 1X Diluent for each test strip to be used for testing. Mix uniformly, but gently. Avoid foaming.

5. TMB Substrate Solution
Ready to use as supplied.
6. Stop Solution
Ready to use as supplied.
7. Microtiter Plate
Ready to use as supplied. Unseal Microtiter Pouch and remove plate from pouch. Remove all strips and wells that will not be used in the assay and place back in pouch and re-seal along with desiccant.
8. Dog PONT Calibrator
Add 1.0 mL of distilled or de-ionized water to the lyophilized Dog PONT Calibrator and mix gently until dissolved. The calibrator is now at a concentration of 200 ng/mL (**the reconstituted calibrator should be aliquoted and frozen if future use is intended**). Dog PONT Calibrators need to be prepared immediately before use (see chart below). Mix well between each step. Avoid foaming.

Calibrator	Concentration (ng/mL)	Calibrator Volume added to 1X Diluent	Volume of 1X Diluent
6	10	30 μ L Dog PONT Calibrator	570 μ L
5	5	0.3 mL Calibrator 6	0.3 mL
4	2.5	0.3 mL Calibrator 5	0.3 mL
3	1.25	0.3 mL Calibrator 4	0.3 mL
2	0.625	0.3 mL Calibrator 3	0.3 mL
1	0.3125	0.3 mL Calibrator 2	0.3 mL
0	0		0.6 mL

STORAGE AND STABILITY

1. Complete Kit
The expiration date for the kit is stated on the outer label. The recommended storage temperature is 4°C. **Note: See long term storage recommendations below for the Dog PONT Calibrator.**
2. Diluent
The Diluent solution is stable until the expiration date and should be stored at 4°C.
3. Wash Solution
The 20X Wash Solution Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions can be stored at room temperature (RT, 16-25°C) or at 4°C.
4. Detection Antibody 100X
Undiluted Biotin conjugated anti-PONT should be stored at 4°C and diluted immediately prior to use. The working conjugate solution is stable for up to 1 hour when stored in the dark.
5. HRP-Streptavidin 100X
Undiluted horseradish peroxidase conjugated streptavidin should be stored at 4°C and diluted immediately prior to use. The working conjugate solution is stable for up to 1 hour when stored in the dark.
6. TMB Substrate Solution
The TMB Substrate Solution should be stored at 4°C and is stable until the expiration date.
7. Stop Solution
The Stop Solution should be stored at 4°C and is stable until the expiration date.
8. Microtiter Plate
Anti-dog PONT coated wells are stable until the expiration date and should be stored at 4°C in the sealed foil pouch with a desiccant pack.
9. Dog PONT Calibrator
The lyophilized Dog PONT calibrator should be stored at 4°C or frozen until reconstituted. The reconstituted calibrator should be aliquoted out and stored frozen (Avoid multiple freeze-thaw cycles). The working calibrator solutions should be prepared immediately prior to use.

INDICATIONS OF INSTABILITY

If the test is performing correctly, the results observed with the calibrator solutions should be within 20% of the expected values.

SPECIMEN COLLECTION AND HANDLING

Blood should be collected by venipuncture and the serum separated from the cells, after clot formation, by centrifugation. For plasma samples, blood should be collected into a container with an anticoagulant and then centrifuged. Care should be taken to minimize hemolysis, excessive hemolysis can impact your results. Assay immediately or aliquot and store samples at -20°C. Avoid repeated freezing/thawing.

For any sample that might contain pathogens, care must be taken to prevent contact with open wounds. No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.

ASSAY PROTOCOL

Dilution of Samples

The assay for quantification of PONT in samples requires that each test sample be diluted before use. For a single step determination a dilution of 1:20 is appropriate for most plasma samples. A dilution of 1:100 is appropriate for most urine samples. For absolute quantification of samples that yield results outside the range of the calibration curve, a lesser or greater dilution might be required. If unsure of sample level, a serial dilution with one or two representative samples before running the entire plate is highly recommended.

To prepare a 1:20 dilution of sample, transfer 20 μ L of sample to 380 μ L of 1X Diluent. This gives you a 1:20 dilution. Mix thoroughly.

To prepare a 1:100 dilution of sample, transfer 5 μ L of sample to 495 μ L of 1X Diluent. This gives you a 1:100 dilution. Mix thoroughly.

Procedure

1. Bring all reagents to RT before use.
2. Pipette 100 μ L of
 - Calibrator 0 (0.0 ng/mL) in duplicate
 - Calibrator 1 (0.3125 ng/mL) in duplicate
 - Calibrator 2 (0.625 ng/mL) in duplicate
 - Calibrator 3 (1.25 ng/mL) in duplicate
 - Calibrator 4 (2.5 ng/mL) in duplicate
 - Calibrator 5 (5 ng/mL) in duplicate
 - Calibrator 6 (10 ng/mL) in duplicate
3. Pipette 100 μ L of diluted sample (in duplicate) into pre-designated wells.
4. Incubate the Microtiter Plate at 22°C (RT) for 120 \pm 2 minutes while shaking on an orbital shaker. Keep plate covered and level during incubation.
5. Following incubation, aspirate the contents of the wells.
6. Completely fill each well with appropriately diluted Wash Solution and aspirate. Repeat three times, for a total of four washes. If washing manually; completely fill wells with wash buffer, invert the plate and pour/shake out the contents in a waste container. Follow this by sharply striking the wells on absorbent paper to remove residual buffer. Repeat 3 times for a total of 4 washes.
7. Pipette 100 μ L of appropriately diluted detection antibody to each well. Incubate at 22°C (RT) for twenty (20 \pm 2) minutes while shaking on an orbital shaker. Keep plate covered in the dark and level during incubation.
8. Wash and blot the wells as described in Steps 5 and 6.

9. Pipette 100 μ L of appropriately diluted HRP-streptavidin to each well. Incubate at 22°C (RT) for twenty (20 \pm 2) minutes while shaking on an orbital shaker. Keep plate covered in the dark and level during incubation.
10. Wash and blot the wells as described in Steps 5 and 6.
11. Pipette 100 μ L of TMB Substrate Solution into each well.
12. Incubate in the dark at RT for precisely ten (10) minutes while shaking on an orbital shaker.
13. After ten (10) minutes, add 100 μ L of Stop Solution to each well.
14. Determine the absorbance at 450 nm of the contents of each well. Calibrate reader to manufacturer's specifications.

The absorbance of the final reaction mixture can be measured up to 2 hours after the addition of the Stop Solution. However, good laboratory practice dictates that the measurement be made as soon as possible.

RESULTS

1. Subtract the average background value from the test values for each sample.
2. Using the results observed for the calibrators construct a calibration curve. The appropriate curve fit is that of a four-parameter logistics curve. A second order polynomial (quadratic) or other curve fits may also be used.
3. Interpolate test sample values from calibration curve. Correct for sample dilution factor to arrive at PONT concentration in original sample.

QUALITY CONTROL

In accord with good laboratory practice, the assays for specific PONT require meticulous quality control. Each laboratory should use routine quality control procedures to establish inter- and intra-assay precision and performance characteristics.

LIMITATION OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the information contained in the package insert instructions and with adherence to good laboratory practice.
2. Factors that might affect the performance of the assay include proper instrument function, cleanliness of glassware, quality of distilled or de-ionized water, and accuracy of reagent and sample pipettings, washing technique, incubation time or temperature.

FOR RESEARCH USE ONLY

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