INTRODUCTION

Paraoxonase 1 (PON1) is an enzyme that is synthesized in liver and secreted into blood, where it associates with HDL. It hydrolyses organophosphates but its natural substrates are currently unknown. It has been reported that PON1 levels, as measured by enzyme activity, decrease in cats with feline infectious peritonitis (ref 1).

PRINCIPLE OF THE ASSAY

The assay uses polyclonal antibodies generated against recombinant cat PON1. Standards and dissociated/diluted samples (100 µl) are incubated in anti-PON1 coated microtiter wells for 45 minutes. After washing the wells, HRP-conjugate (100 µl) is added and incubated for 45 minutes. If PON1 molecules are present, they are sandwiched between the capture and detection antibodies. The wells are then washed to remove unbound HRP-conjugate. TMB is added and incubated for 20 minutes. If PON1 is present, a blue color develops. Color development is stopped after 20-minutes by addition of Stop Solution, changing the color to yellow. Absorbance is measured at 450 nm. The concentration of PON1 is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-PON1 coated plate (12 x 8-well strips)
- HRP conjugate stock.
- PON1 stock, 1 vial. **Store at -20°C**
- 20x Wash Solution: TBS50-20, 50 ml
- Dissociation Buffer: DC10-1, 1 x 10 ml
- Diluent: SB60-1, 1 x 60 ml
- TMB: TMB11-1, 11 ml
- Stop Solution: SS11-1, 11 ml

Materials required but not provided:

- Pipettors and tips
- Distilled or deionized water
- Polypropylene tubes or 96-well polystyrene plates
- Vortex mixer
- Absorbent paper or paper towels
- Plate incubator/shaker
- Plate washer
- Plate reader capable of measuring absorbance at 450 nm
- Graphing software

STORAGE

Store the standard stock vial at -20°C. The remainder of the kit should be stored at 4°C and the microtiter plate should be kept in a sealed bag with desiccant. The kit will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature before use.
2. Reliable and reproducible results will be obtained when the assay is conducted with a complete understanding of the instructions and with adherence to good laboratory practice.
3. It is important that standards and samples be added to the ELISA plate quickly. If testing large numbers of samples, rather than pipetting standards and samples from individual tubes into the ELISA plate, we recommend the following: pipette an excess volume of standards and samples into wells of a blank polystyrene 96-well plate[1]. Then use an 8 or 12-channel multi-pipettor to quickly transfer 100 µl aliquots to the wells of the antibody-coated plate.
4. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
5. Laboratory temperature will influence absorbance readings. The assay was calibrated using a shaking incubator set at 150 rpm and 25°C. Performing the assay at lower temperatures and mixing speeds may result in lower absorbance values.

WASH SOLUTION

The wash solution is provided as a 20x stock. Prior to use, dilute the contents of the bottle (50 ml) with 950 ml of distilled or deionized water. Unused wash buffer may be stored at 4°C for one week.

DISSOCIATION BUFFER

The dissociation buffer (DC10-1) is used for the initial 10-fold dilution of samples as described in the “SAMPLES” section. It is supplied ready to use. DO NOT substitute other buffers.

DILUENT

The diluent (SB60-1) is formulated for measurement of PON1 in serum. It is used for further dilution of dissociated samples, and for dilution of the HRP conjugate stock. It is supplied ready to use. DO NOT substitute other buffers.

[1] Standards and sample dilutions may also be prepared directly in a blank polystyrene plate.

STANDARD

1. The stock is lyophilized. It is comprised of recombinant cat PON1 in a stabilizing matrix. Reconstitute it with deionized water as described on the vial label and gently mix. Prepare the 20 ng/ml standard as described on the label.
2. Label seven polypropylene tubes as 10, 5, 2.5, 1.25, 0.625, 0.313 and 0 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 20 ng/ml PON1 standard into the tube labeled 10 ng/ml and mix. This provides the 10 ng/ml PON1 standard.
4. Similarly prepare the 5 to 0.313 ng/ml standards by two-fold serial dilution.

IMPORTANT – If future use of the stock is intended, the capped vial must be frozen at or below -20°C within 30 minutes of reconstitution.

SAMPLES

The assay is intended for measurement of PON1 in cat serum. Because PON1 associates with HDL, which interferes with antibody binding, samples must first be treated with Dissociation Buffer DC10-1 to dissociate PON1 from HDL. After 15 min, dissociated samples are further diluted with Diluent SB60-1.

We found PON-1 levels ranging from 3 to 25 μg/ml in cat serum. The sample preparation procedure described below worked well for all samples we tested.

Dissociation Step

1. Mix 5.0 μl of serum with 45 μl of Dissociation Buffer DC10-1. This can be done in microcentrifuge tubes or wells of a blank 96-well polystyrene plate (not provided). This provides a 10-fold dilution of the sample.
2. Incubate at room temperature for 15 minutes.

Further Dilution Step

We found that a final dilution of 1600-fold worked well for most samples. A 1600-fold dilution may be obtained as follows.

1. Dispense 318 μl of SB60-1 Diluent into a microcentrifuge tube or a well of a blank 96-well polystyrene plate.
2. Using a precision pipettor, mix 2.0 μl of the dissociated sample (above) with 318 μl of SB60-1. This provides a 1600-fold dilution.

If further dilution is required, dilute the 1600-fold sample with SB60-1 as appropriate. Final dilutions less than 1600-fold (i.e., 800-fold) should not be used because matrix effects may occur.

HRP CONJUGATE

The HRP conjugate is provided as a concentrated stock. Prior to use it must be diluted with Diluent SB60-1 as described on the vial label. Use 100 μl of the diluted HRP conjugate per well.

PROCEDURE

1. Secure the desired number of 8-well strips in the cassette. Unused strips should be stored in a sealed bag with desiccant at 4°C.
2. Dispense 100 μl of standards and dissociated/diluted samples into appropriate wells. We recommend that standards and samples be tested in duplicate.
3. Incubate on a plate shaker at 150 rpm and 25°C for 45 minutes.
4. Empty and wash the microtiter wells 5 times with 1x Wash Solution using a plate washer (400 μl/well).
5. Dispense 100 μl of diluted HRP conjugate into the wells.
6. Incubate on a plate shaker at 150 rpm and 25°C for 45-minutes.
7. Empty and wash the microtiter wells 5 times with 1x Wash Solution using a plate washer (400 μl/well).
8. If necessary, strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
9. Dispense 100 μl of TMB into each well.
10. Incubate on an orbital micro-plate shaker at 150 rpm at 25°C for 20 minutes.
11. After 20 minutes stop the reaction by adding 100 μl of Stop Solution to each well.
12. Gently mix. It is important to make sure that all the blue color changes to yellow.
13. Read absorbance at 450 nm[2] with a plate reader within 5 minutes.

RESULTS

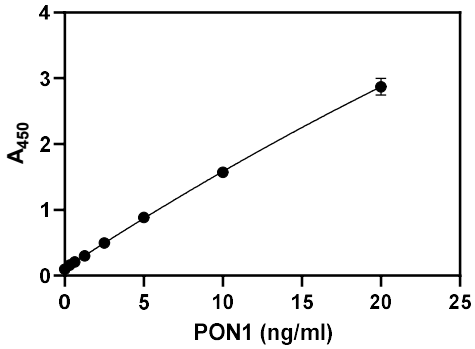
1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus the PON1 concentration. We suggest fitting data second order polynomial equation.
2. Derive the concentration of PON1 in the samples.
3. Multiply the derived concentration by the dilution factor to determine the concentration in the original sample.
4. If the absorbance values of samples fall outside the standard curve, samples should be further diluted appropriately and re-tested.

[2] If absorbance of the high standard is ≥4 when measured at 450 nm, absorbance of all standards and samples should be read at 405 nm.

TYPICAL STANDARD CURVE

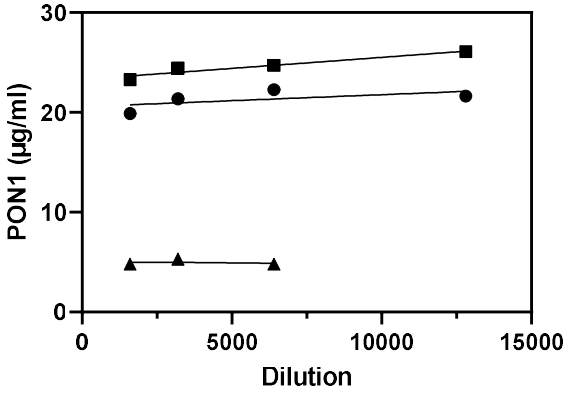
A typical standard curve is shown below. This curve is for illustration only.

PON1 (ng/ml)	A_{450}
20	2.872
10	1.571
5	0.885
2.5	0.498
1.25	0.303
0.625	0.210
0.313	0.163
0	0.098



PERFORMANCE

Linearity: To assess the linearity of the assay, serum samples with PON1 concentrations of 24.4, 21.3 and 4.9 μg/ml were treated with dissociation buffer and then serially diluted with SB60-1 to give values within range of the assay.



REFERENCES

1. Tecles F, Caldin M, Tvarijonaviciute A, Escribano D, Martinez-Subiela S, and Ceron JJ. Serum Biomarkers of oxidative stress in cats with feline infectious peritonitis. Research in Veterinary Science. 100 (2015) 12-17. http://dx.doi.org/10.1016/j.rvsc.2015.02.007

Rev 080723

For technical assistance please email us: techsupport@lifediagnostics.com

Life Diagnostics, Inc.
124 Turner Lane, West Chester, PA 19380
610-431-7707 - info@lifediagnostics.com.com – www.lifediagnostics.com