Dog Cystatin B ELISA CYSB-4

INTRODUCTION

Cystatin B is a biomarker of chronic and acute kidney injury in dogs (refs 1 & 2). During chronic kidney disease (CKD), levels in urine can reach 500 ng/ml or greater. At Life Diagnostics we found levels of approximately 100 ng/ml in serum from dogs with CKD.

PRINCIPLE OF THE ASSAY

The assay uses affinity purified polyclonal antibodies against recombinant dog Cystatin B that were developed at Life Diagnostics. One is used as coating antibody. The other is conjugated to HRP and used for detection. Standards and diluted samples (100 µl) are incubated in the coated microtiter wells for 45 minutes. After washing the wells, HRP-conjugate (100 µl) is added and incubated for 45 minutes. If Cystatin B 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 Cystatin B 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 Cystatin B is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-Cystatin B coated plate (12 x 8-well strips)
- Anti-Cystatin B HRP stock
- Cystatin B stock, 2 vials. **Store at -20°C**
- 20x Wash Solution: TBS50-20, 50 ml
- Diluent: SB50-1, 2 x 50 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 vials 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. 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.
3. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
4. 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.

DILUENT

The Diluent (SB50-1) is formulated for measurement of Cystatin B in serum. It is supplied ready to use. DO NOT substitute other buffers.

STANDARD

1. The stock is lyophilized. It is comprised of recombinant dog Cystatin B in a stabilizing matrix. Reconstitute it with deionized water as described on the vial label and gently mix. Prepare the 40 ng/ml standard as described on the label.
2. Label seven polypropylene tubes as 20, 10, 5, 2.5, 1.25, 0.625 and 0 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 40 ng/ml Cystatin B standard into the tube labeled 20 ng/ml and mix. This provides the 20 ng/ml Cystatin B standard.
4. Similarly prepare the remaining standards by two-fold serial dilution.

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

HRP CONJUGATE

The HRP conjugate is provided as a concentrated stock. Dilute the stock with SB50-1 diluent as described on the vial label right before use.

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

SAMPLES

The assay is intended for measurement of Cystatin B in dog serum or urine. Optimal dilutions should be determined empirically. To avoid matrix effects, serum and urine should be diluted at least 10-fold with diluent SB50-1.

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 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 with a plate reader within 5 minutes.

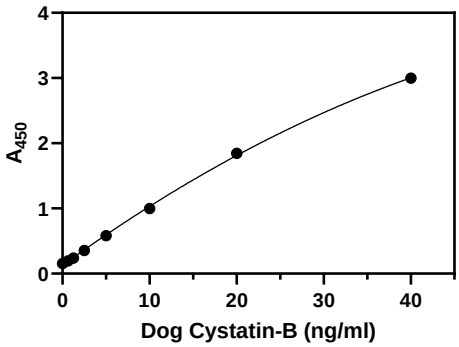
RESULTS

1. Using curve fitting software, graph the absorbance values of the standards on the Y-axis versus Cystatin B concentration on the X-axis.
2. Fit the curve to a second order polynomial (quadratic) equation and derive the concentration of Cystatin B in the diluted samples.
3. Multiply derived values by the dilution factor(s) to determine Cystatin B concentration in the original sample.
4. If the absorbance values of diluted samples fall outside the standard curve, samples should be further diluted and re-tested.

TYPICAL STANDARD CURVE

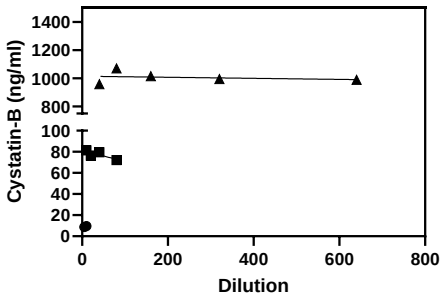
A typical standard curve is shown below. This curve is for illustration only.

Cystatin B (ng/ml)	A_{450}
40	2.998
20	1.844
10	1.000
5	0.581
2.5	0.355
1.25	0.240
0.625	0.195
0	0.155



PERFORMANCE

Linearity: To assess the linearity of the assay, healthy dog urine was spiked with Cystatin B at concentrations of 10, 100 and 1000 mg/ml and serially diluted with SB50-1 to give values within range of the assay.



REFERENCES

1. Segev G. et al. Urinary cystatin B differentiates progressive versus stable IRIS stage 1 chronic kidney disease in dogs. J Vet Intern Med. 2023;37:2251-2260
2. Gordin E. et al. A clinical study on urinary clusterin and cystatin B in dogs with spontaneous acute kidney injury. Vet. Sci. 2024, 11, 200. https://doi.org/10.3390/vetsci11050200

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