# Serum concentrations of pepsinogen A in healthy dogs after food deprivation and after feeding

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**Objective**—To develop and validate an ELISA for measurement of serum canine pepsinogen A (cPG A) as a diagnostic marker of gastric disorders in dogs and to measure serum cPG A in healthy dogs after food deprivation and after feeding.

**Sample Population**—Sera from 72 healthy dogs.

**Procedure**—A sandwich ELISA was developed and validated. The reference range for serum concentrations of cPG A was determined in 64 healthy dogs. Postprandial changes in serum concentrations of cPG A were evaluated in 8 healthy dogs.

**Results**—Assay sensitivity was 18 µg/L, and the maximum detectable concentration was 1,080 µg/L. The observed-to-expected ratio (O:E) for 3 serial dilutions of 3 serum samples ranged from 69.3 to 104.1%. The O:E for 3 serum samples spiked with 8 concentrations of cPG A ranged from 58.8 to 120.4%. Coefficients of variation for intra- and interassay variability of 3 serum samples ranged from 7.6 to 11.9% and from 10.1 to 13.1%, respectively. Mean ± SD serum concentration of cPG A in healthy dogs was 63.8 ± 31.0 µg/L and the reference range was 18 to 129 µg/L. Significant increases in serum concentrations of cPG A were observed between 1 and 7 hours after feeding.

**Conclusions and Clinical Relevance**—The ELISA for measuring cPG A was sufficiently sensitive, linear, accurate, precise, and reproducible for clinical use. Serum concentrations of cPG A increase substantially after feeding, which should be taken into account when conducting clinical studies. (*Am J Vet Res* 2003; 64:1146–1150)

Gastric diseases in general, and gastritis in particular, are common causes for illness in dogs. Although there are specific indicators for diseases of the liver, pancreas, or kidneys, none are available for gastritis. Currently, establishing a definitive diagnosis of gastritis requires invasive and expensive diagnostic tests (ie, gastroscopy). Therefore, a minimally invasive, inexpensive, sensitive, and specific marker for gastritis in dogs would be desirable.

Measurement of **pepsinogen (PG)** in serum has been used for the diagnosis of various gastric and duodenal disorders in human beings and other animals.[1-5] Pepsinogen is the precursor of pepsin, an aspartic proteinase synthesized mainly by gastric chief cells. Pepsinogen is heterogeneous with 2 main groups identified in many species (ie, PG A and PG C).[6-8] Each group is synthesized in specific areas of the gastrointestinal tract. In humans, PG A and PG C are synthesized in the gastric fundus; in addition, PG C is also synthesized in the pyloric region and duodenum. This difference in the cellular location between PG A and C and the development of specific immunoassays for both groups of PG has been useful in the diagnosis of various gastric and duodenal disorders in humans (eg, gastric and duodenal ulcers, gastritis, and gastric adenocarcinoma).[2]

In contrast, only catalytic assays have been used in veterinary medicine to estimate peptic activity in serum. These assays have been used as a tool to diagnose ostertagiosis in cattle[4] and gastric ulcers in foals.[5] An increase in serum peptic activity in cattle reflects the development and emergence of larval stages of *Ostertagia ostertagi* with subsequent mucosal damage,[4] whereas serum peptic activity is significantly increased in foals with gastric or duodenal ulcers.[5]

Indirect catalytic assays such as the ones used in veterinary medicine can only detect pepsin, the active form of PG; thus, they require activation of the zymogen. Therefore, a potential problem of catalytic assays is the additional activation of other proteinases in serum that may lead to erroneous measurements of peptic activity. In contrast, immunoassays measure the concentration of protein by detecting antigenic determinants on the protein surface that are shared with the zymogen; therefore, they allow more accurate quantification of the zymogen.

In a study[9] conducted by our laboratory group, we reported the purification of various isoforms of **canine PG (cPG)** A, but we were unable to purify PG C from the gastric tissues of dogs. In another study,[10] investigators were unable to isolate cDNA encoding cPG C, indicating that PG C may not be expressed in dogs. Therefore, it is believed that PG A is the predominant type of PG in dogs.[9,10]

An immunoassay for measurement of cPG A concentrations may be a clinically useful, minimally invasive diagnostic tool for use in classifying gastric disorders in dogs. The purposes of the study described here were to develop and validate an ELISA for measurement of serum concentrations of cPG A as a potential diagnostic marker for gastric disorders in dogs.

Received January 24, 2003.
Accepted March 18, 2003.
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Supported by the Morris Animal Foundation.
Presented in part at the 2002 Forum of the American College of Veterinary Internal Medicine, Dallas, May 2002.
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Additionally, another objective of the study was to describe the influence of feeding on serum concentrations of cPG A in healthy dogs.

## Materials and Methods

**Animals**—Eight adult Beagle dogs (4 males and 4 females) between 2.5 and 3.0 years of age were selected from a colony of laboratory dogs owned by and housed at a commercial company.[a] Physical examination did not reveal abnormalities in any of the dogs. Evaluation of a CBC and serum biochemical analyses also did not reveal major abnormalities.

**Influence of feeding on cPG A concentrations**—Venous access was established in each dog by insertion of a 12-inch-long, 18.5-gauge, indwelling catheter[b] into a jugular vein. Dogs were fed at 10 AM on the day prior to the study. At 3 PM of that day, all bowls and unconsumed food were removed from the enclosures. On the day of the study, a baseline blood sample was collected at 8 AM, and all dogs were then fed a commercial, dry, maintenance diet.[c] Additional blood samples were collected 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240, 300, 360, 420, and 480 minutes after feeding. Serum was separated immediately after clot formation. Serum samples were frozen at –80°C, shipped on dry ice, and maintained frozen at –80°C until analyzed. Serum concentrations of cPG A were measured by use of a cPG A ELISA.

**Preparation of antibodies**—The cPG A was purified from gastric tissues obtained from dogs, as reported elsewhere.[9] Two sheep were inoculated with 100 µg of cPG A mixed with 1 mg of saponin[d] as an adjuvant. Sheep were reinoculated with cPG A mixed with the adjuvant every 3 weeks for a total of 6 inoculations. An affinity chromatography column[e] was used to purify monospecific polyclonal antibodies from sheep serum. The column was prepared in accordance with the manufacturer's instructions. For lipoprotein precipitation, 25 mL of sheep antiserum was thawed and added to 25 mL of 1M $CaCl_2$ and 1 mL of 10% dextran sulfate solution. This mixture was gently inverted for 15 minutes at 4°C. The mixture was then centrifuged (10,000 × g for 10 minutes at 4°C), and the precipitate was discarded. A gel filtration column[f] was used, and buffer A (75mM Tris HCl, 150mM NaCl [pH, 8.0]) was added to the column in accordance with the manufacturer's instructions. The antiserum in buffer A was then applied to the previously prepared affinity chromatography column mounted on a fast-performance liquid chromatography purification system.[g] Absorbance of the effluent was measured at a wavelength of 280 nm. The column was washed with buffer A until the absorbance had returned to baseline values. Then, the mobile phase was changed to buffer B (100mM glycine, 500mM NaCl [pH, 2.5]), and 1-mL fractions of the effluent were collected in test tubes that contained 300 µL of 1M Tris-HCl (pH, 8.0). Purified antibodies were concentrated by use of a centrifugal filter device,[h] the buffer was changed to PBS solution (pH, 7.2),[i] and the concentration in each tube was adjusted to 1 mg/mL. Aliquots (100 µL) were stored frozen at –20°C.

For biotinylation, 4.5 mg of purified monospecific polyclonal cPG A-antibody in PBS solution was injected into a dialysis cassette,[j] and a 20-fold molar excess of biotin[k] was added. After incubation for 12 hours at room temperature (22°C), the material was dialyzed 3 times against 1,000 mL of PBS solution for 1 hour at 4°C. Biotinylation efficiency was determined by use of a 2-(4'-hydroxyazonbenzene) benzoic acid avidin assay kit.[l] The biotinylation procedure was repeated until we achieved a biotinylation coefficient > 4.0. Biotinylated antibodies were adjusted to a concentration of 1 mg/mL and frozen at –20°C in aliquots of 100 µL.

**Development of the ELISA for cPG-A**—Standard 96-well flat-bottom ELISA plates[m] were coated with 200 ng of affinity-purified anti-cPG A antibodies/well in 100 µL of carbonate-bicarbonate buffer (pH, 9.4). Plates were incubated with mixing for 1 hour at 37°C by use of an automated plate incubator-shaker[n]; plates were then washed 4 times with 300 mL of PBS solution/well by use of an automated plate washer.[o] Remaining binding sites were blocked by addition of 10% bovine serum albumin in PBS solution (200 µL/well). Plates were incubated for 1 hour at 37°C and then washed as described previously. Standard solutions of cPG A (10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, and 0.08 µg/L) were prepared by serial dilution of pure cPG A in buffer C (PBS solution, 1% bovine serum albumin, and 0.05% polyoxyethylenesorbitan monolaurate[p]) and frozen in 300-µL aliquots at –80°C until use. Standard solutions were thawed immediately prior to loading of each plate. Serum samples were prepared in a 1:120 dilution with buffer C. A volume (100 µL/well) of standard solution or sample was loaded in duplicate wells. An equal volume (100 µL) of buffer C was used as a negative-control sample. Plates were incubated for 2 hours at 37°C and then washed as described previously. A solution containing 50 ng of biotinylated anti-cPG A antibodies in 100 µL of buffer C was added to each well. After incubation for 1 hour at 37°C and washing as described previously, 50 ng of horseradish peroxidase-labeled streptavidin[q] in 100 µL of buffer C was added to each well. Plates were again incubated for 1 hour at 37°C and washed as described previously. Retained horseradish peroxidase was developed by the addition of 100 µL of 3,3',5,5'-tetramethylbenzidine solution[r] /well. The reaction was stopped after 12 minutes by the addition of 100 µL of a solution of 4M acetic acid-1 N sulfuric acid/well. Absorbance was measured at 450 nm.[s] Standard curves were calculated for a 4-parameter curve fit by use of the following equation:

$$y = ([A - D]/[1 + \{x/C\}^B]) + D$$

where A is the y-axis value corresponding to the asymptote at low values of the x-axis, D is the y-axis value corresponding to the asymptote at high values of the x axis, C is the x-axis value corresponding to the midpoint between A and D, and B describes how rapidly the curve makes the transition from the asymptotes in the center of the curve. Parameters were calculated by use of an algorithm based on the Levenberg-Marquardt equation.

**Validation of the ELISA**—The ELISA was validated by examination of the assay sensitivity, upper limit of the working range, dilutional parallelism, spiking recovery, and intra- and interassay variability. Assay sensitivity (ie, limit of detection) was defined as the apparent concentration of cPG A that produced an absorbance equal to the mean plus 3 times the SD of the absorbance of 10 duplicates of the negative-control solution (buffer C). The upper limit of the working range was defined as the apparent concentration of cPG A that produced an absorbance equal to the mean minus 3 times the SD of the absorbance of 10 duplicates of a cPG A solution of 100 µg/L. Three serum samples were used to determine dilutional parallelism. All 3 samples were evaluated at dilutions of 1:1 (which represented a serum dilution of 1:60), 1:2, 1:4, and 1:8. Spiking recovery was determined by the addition of cPG A (5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, and 0.04 µg/L) to each of 3 serum samples. Intra-assay variability was determined by evaluating results for each of 3 serum samples assayed 10 times within the same run of the assay. Interassay variability was determined by evaluating results for each of 3 serum samples during 10 consecutive runs of the assay. The reference range was determined on the basis of the central 95th percentile (from

2.5 to 97.5%) for concentrations of cPG A in sera obtained from 64 healthy dogs.

**Statistical analysis**—Variations in concentrations of cPG A over time were analyzed by comparison of serum cPG A immunoreactivity at the various time points relative to baseline concentrations by use of a 1-way, repeated-measures ANOVA, which was followed by the Dunnett multiple-comparison test. Data were analyzed by use of a statistical software package.[1] Significance was assigned for values of $P < 0.05$.

## Results

**ELISA**—Reproducible standard curves were generated by use of the ELISA (**Fig 1**). Mean ± SD absorbance at 450 nm for the 10 duplicates of a negative-control sample was 0.021 ± 0.018. Extrapolation from the standard curve for an absorbance of the mean plus 3 times the SD was equivalent to a cPG A concentration of 0.15 µg/L. Because serum samples were diluted 1:120 prior to assay, the calculated assay sensitivity for serum samples was 18 µg/L. Mean absorbance at 450 nm for the 10 duplicate wells containing 100 µg of cPGA/L was 2.805 ± 0.275. Extrapolation from the standard curve for an absorbance of the mean minus 3 times the SD was equivalent to a cPG A concentration of 9 µg/L, which corresponded to a maximum concentration in serum of 1,080 µg/L.

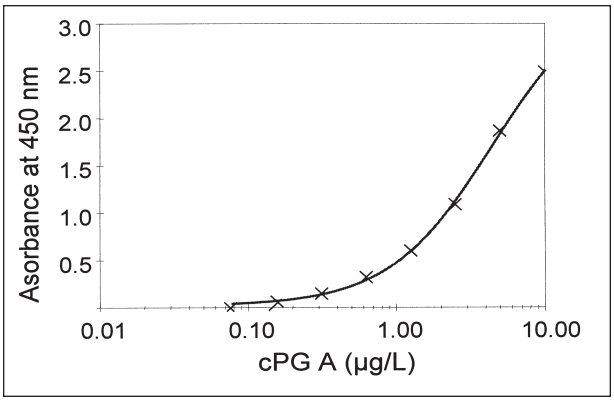


Figure 1—Representative standard curve obtained for an ELISA that measured canine pepsinogen A (cPG A). The curve was generated by a computer software package that used a 4-parameter curve fit. Parameters were calculated by use of an algorithm based on the Levenberg-Marquardt equation as follows: $y = ([0.020 - 1.243]/[1 + \{x/0.222\}^{3.422}]) + 1.243$.

Table 1—Dilutional parallelism of serum concentrations of canine pepsinogen A (cPG A) for each of 3 serum samples

| Sample | Dilution* | Observed (µg/L) | Expected (µg/L) | O:E (%) |
|---|---|---|---|---|
| 1 | 1:1 | 45.24 | NA | NA |
| | 1:2 | 23.1 | 22.7 | 101.7 |
| | 1:4 | 11.8 | 11.4 | 104.1 |
| | 1:8 | 5.5 | 5.7 | 97.2 |
| 2 | 1:1 | 67.1 | NA | NA |
| | 1:2 | 32.4 | 33.5 | 96.6 |
| | 1:4 | 17.2 | 16.8 | 102.3 |
| | 1:8 | 5.9 | 8.4 | 70.8 |
| 3 | 1:1 | 141.2 | NA | NA |
| | 1:2 | 65.0 | 70.6 | 92.0 |
| | 1:4 | 31.9 | 35.3 | 90.2 |
| | 1:8 | 12.2 | 17.7 | 69.3 |

*Samples were diluted in buffer C. The 1:1 dilution represents a serum dilution of 1 in 60. All samples were evaluated in duplicate, and results were multiplied by 60 to reflect the serum concentrations of cPG A that are reported here.
O:E = Ratio of observed concentration to expected concentration. NA = Not applicable.

Linearity of the assay was determined by evaluating dilutional parallelism. Ratios of observed results to expected results for each of 3 serum samples at 4 dilutions ranged from 69.3 to 104.1% (mean, 91.6 ± 13.0%; **Table 1**). Accuracy of the assay was evaluated by determining spiking recovery. Ratios of observed results to expected results for each of 3 serum samples and 8 spiking concentrations ranged from 58.8 to 120.4% (mean, 86.5 ± 14.9%) when we assumed recovery of 70% (**Table 2**).

Intra-assay variability for 3 serum samples ranged from 7.6 to 11.9% (mean, 9.5 ± 2.2%). The coefficient of variation for the 3 samples (mean ± SD concentrations of 40.6 ± 3.7, 83.8 ± 10.0, and 257.9 ± 7.6 µg/L) was 9.1, 11.9, and 7.6%, respectively. Interassay variability for 3 serum samples ranged from 10.1 to 13.3% (mean, 11.8 ± 1.6%). The coefficient of variation for the 3 samples (mean ± SD concentrations of 52.8 ± 6.9, 90.2 ± 9.1, and 126.2 ± 15.5 µg/L) was 13.1, 10.1, and 12.3%, respectively.

Mean serum concentration of cPG A for the 64 healthy dogs was 63.8 ± 31.0 µg/L. The calculated reference range (central 95th percentile) was 18 to 129 µg/L.

**Influence of feeding**—All 8 dogs rapidly and completely consumed the meal fed at the onset of the experiment. All dogs had similar postprandial alterations in cPG A concentrations. Baseline serum con-

Table 2—ELISA results for recovery* of cPG A added to each of 3 serum samples

| Sample | Spiking concentration (µg/L) | Observed (µg/L) | Expected (µg/L) | O:E (%) |
|---|---|---|---|---|
| 1 | 0.00 | 0.46 | NA | NA |
| | 0.04 | 0.58 | 0.48 | 120.4 |
| | 0.08 | 0.48 | 0.51 | 94.6 |
| | 0.16 | 0.53 | 0.57 | 94.3 |
| | 0.31 | 0.70 | 0.68 | 103.6 |
| | 0.63 | 0.89 | 0.89 | 99.7 |
| | 1.25 | 1.09 | 1.33 | 81.5 |
| | 2.50 | 1.91 | 2.21 | 86.4 |
| | 5.00 | 2.33 | 3.96 | 58.8 |
| 2 | 0.00 | 0.52 | NA | NA |
| | 0.04 | 0.53 | 0.55 | 97.6 |
| | 0.08 | 0.56 | 0.57 | 97.4 |
| | 0.16 | 0.61 | 0.63 | 96.4 |
| | 0.31 | 0.65 | 0.74 | 88.2 |
| | 0.63 | 0.69 | 0.96 | 72.0 |
| | 1.25 | 1.02 | 1.39 | 73.2 |
| | 2.50 | 1.66 | 2.27 | 73.1 |
| | 5.00 | 2.67 | 4.02 | 66.4 |
| 3 | 0.00 | 0.98 | NA | NA |
| | 0.04 | 0.89 | 1.01 | 88.0 |
| | 0.08 | 1.11 | 1.04 | 107.1 |
| | 0.16 | 1.08 | 1.09 | 98.4 |
| | 0.31 | 0.98 | 1.20 | 81.4 |
| | 0.63 | 1.04 | 1.42 | 73.1 |
| | 1.25 | 1.38 | 1.86 | 74.4 |
| | 2.50 | 1.86 | 2.73 | 68.0 |
| | 5.00 | 3.64 | 4.48 | 81.2 |

*Assumed recovery was 70% for all experiments.

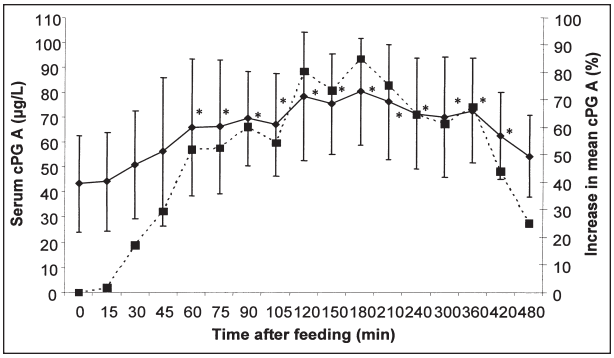


Figure 2—Mean ± SD serum concentrations of cPG A (diamonds) in 8 healthy dogs and percentage increase relative to the baseline value (squares) at various time points after feeding. *Concentration differs significantly ($P < 0.001$) from the baseline concentration.

centrations of cPG A for all 8 dogs were within the reference range (group mean, 43.4 ± 19.3 µg/L). Mean serum concentrations of cPG A were significantly ($P < 0.001$) higher 60 minutes after feeding (65.9 ± 27.4 µg/L; 52% above baseline value), compared with the mean baseline value (**Fig 2**). Mean serum cPG A concentration reached a peak 180 minutes after feeding (80.3 ± 21.4 µg/L; 85% above baseline value). Mean serum cPG A concentration 480 minutes after feeding (54.3 ± 16.4 µg/L; 25% above baseline value) did not differ significantly from the mean baseline value. Serum cPG A concentration did not exceed the reference range in any of the 8 dogs at any time point.

## Discussion

Evaluation of dilutional parallelism of the ELISA indicated acceptable linearity for clinical use. The observed-to-expected ratio for 2 samples was 70.8 and 69.3%, respectively, which may be considered unacceptable for clinical application. However, these results were obtained at a dilution of 1:8 and yielded values at the lower end of the working range of the assay. If, as hypothesized dogs with gastritis have serum concentrations of cPG A above the upper area of the working range, a lower degree of linearity for values in the lower area of the working range should not influence the diagnostic performance of this assay.

Analysis of results of spiking recovery revealed that acceptable accuracy of the assay was obtained when we assumed a recovery of 70%. The percentage recovery continuously decreased as a larger concentration of cPG A was added to the samples. This would suggest that some of the added cPG A was rendered undetectable. The mechanism for this aberration is unknown. A possible explanation would be that cPG A is bound by serum proteins that render the molecule undetectable. Other possible causes of this finding that could be considered are substrate exhaustion or stereotactic inhibition of antigen-antibody interaction. However, because the decrease of recovery was consistent among samples, the assay can be considered sufficiently accurate for clinical use.

Analysis of the coefficient of variation for intraassay variability indicated that the assay is precise. The coefficient of variation for interassay variability revealed that the assay is reproducible over the working range.

During the interdigestive phase, PG is synthesized and stored in granules of the chief cells. Feeding stimulates release of PG into the gastric lumen mediated through nervous (eg, cholinergic agents) and hormonal (eg, gastrin) stimuli.[11] A potential change in serum cPG A immunoreactivity after feeding may affect clinical interpretation of the assay. Therefore, the second objective of the study was to evaluate postprandial alterations in serum concentrations of cPG A.

The influence of feeding on serum concentrations of PG has been evaluated in human beings.[12-15] In 2 studies,[13,15] investigators were unable to document significant effects of a test meal on serum concentrations of PG A measured 30, 120, and 210 minutes after the meal was consumed. In another study,[14] a test meal induced a slight and clinically nonsignificant increase in serum concentrations of PG A for up to 180 minutes. In contrast to those findings, yet another study[12] revealed a postprandial increase in serum concentrations of PG A with a maximum increase of 107.6% over baseline concentrations at 30 minutes after eating. Serum concentrations of PG A gradually decreased thereafter and reached baseline values within 2 hours after eating. Postprandial alterations observed in dogs in the study reported here differ from those observed in humans. A significant difference in serum concentrations of cPG A, compared with baseline concentrations, was seen 60 minutes after feeding, with a maximum peak 180 minutes after feeding (85% above baseline value). Furthermore, a significant ($P < 0.001$) increase in serum concentration of cPG A was observed between 1 and 7 hours after feeding.

We do not have an obvious explanation for these differences between dogs and human beings. Because PG A appears to be the predominant isoform of PG in dogs,[9,10] a prolonged postprandial increase in the concentration of PG A may be attributable to a higher initial output of PG A in dogs in response to a meal, compared to the response in humans. Another possibility is species-specific differences in the metabolism of PG. Studies[2,16] in humans documented that despite a negative net charge and a molecular mass of 42 kd, human PG A is almost freely filtered through the glomerular basement membrane; therefore, human PG A is found in urine at concentrations 10 to 100 times higher than concentrations in serum. In addition, PG A is partially metabolized by the kidneys.[17] Thus, it is possible that species-specific differences in the metabolism and mechanisms of excretion of PG A could affect the concentration of PG A in serum. Additional studies are needed to prove this hypothesis.

For the 8 healthy dogs used in the study, serum concentrations of cPG A did not exceed the upper limit of the reference range at any time point. Serum concentrations of cPG A had returned to values statistically similar to baseline values by 8 hours after feeding, although at this time point, mean serum concentration of cPG A was still approximately 25% increased above the mean baseline value. It is unknown how feeding will influence serum concentrations of cPG A in dogs with gastric disease, especially dogs with concentrations close to the upper limit of the reference range. Therefore, it is suggested that food should be withheld

for at least 8, and preferably 12, hours before obtaining samples to determine baseline serum concentrations of cPG A in dogs.

[a]Hill's Science and Technology Center, Topeka, Kan.
[b]Venocath, Abbott Laboratories, Abbott Park, Ill.
[c]Science Diet, Canine Maintenance, Hill's Pet Nutrition Inc, Topeka, Kan.
[d]Quill A, Accurate Chemicals, Westbury, NY.
[e]HI Trap, Amersham Biosciences, Piscataway, NJ.
[f]PD-10, Sephadex G-25 M column, Amersham Biosciences, Piscataway, NJ.
[g]FPLC-system ÄKTApurifier, Amersham Biosciences, Piscataway, NJ.
[h]Centripep YM-10, Amicon, Bedford, Mass.
[i]PBS, Pierce Chemical Co, Rockford, Ill.
[j]Slide-A-Lyzer, 10K dialysis cassette, Pierce Chemical Co, Rockford, Ill.
[k]EZ-Link Sulfo-NHS-LC biotin, Pierce Chemical Co, Rockford, Ill.
[l]ImmunoPure HABA assay kit, Pierce Chemical Co, Rockford, Ill.
[m]Combiplates 8, Labsystems, Helsinki, Finland.
[n]Stat Fax-2200, Awareness Technology Inc, Palm State, Fla.
[o]Columbus microplate washer, Tecan, Salzburg, Austria.
[p]Tween-20, Pierce Chemical Co, Rockford, Ill.
[q]Immunopure streptavidin HRP-conjugated, Pierce Chemical Co, Rockford, Ill.
[r]TMB substrate kit, No. 34021, Pierce Chemical Co, Rockford, Ill.
[s]UV MAX ELISA plate reader and SOFTMAX PRO analysis software package, Molecular Devices, Sunnyvale, Calif.
[t]GraphPad Prism 3.0, GraphPad Software Inc, San Diego, Calif.

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